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Dr. David F. Mettrick and Dr. Sherwin S. Desser
Editors, The Journal of Parasitology
Department of Zoology
University of Toronto
Toronto, Ontario
Canada M5S 1A1

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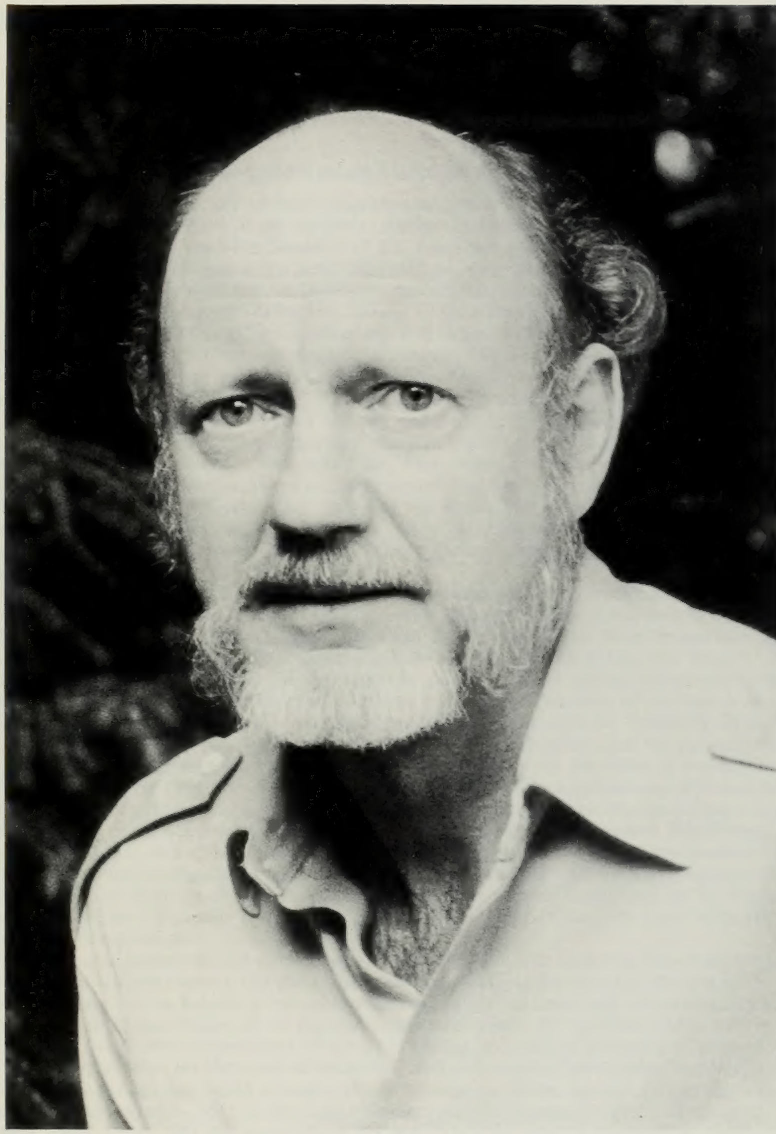

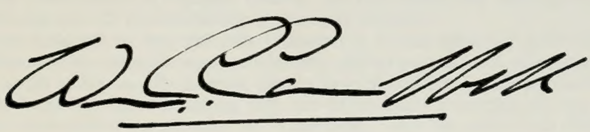

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PRESIDENTIAL ADDRESS*

William C. Campbell
Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, New Jersey 07065

Introduction of President Campbell, by Charles W. Kim

Members of the American Society of Parasitologists and guests, I am very pleased to welcome you to this session of the Presidential Address of the American Society of Parasitologists. This is a personal honor and privilege for me to introduce the 64th President of the American Society of Parasitologists, Dr. William C. Campbell, who has been a colleague and a personal friend for more than 20 years.

Before elaborating on his professional and academic accomplishments, I would like to highlight his personal background. William Cecil Campbell was born on 28 June 1930, in Londonderry, Northern Ireland, and received his early education in County Donegal. He received the B.A. degree with first class honors in 1952 from Trinity College of the University of Dublin with a major in Zoology and a minor in Botany. He then came to the United States to pursue graduate studies at the University of Wisconsin, earning the M.S. degree in 1954 in Veterinary Science and Zoology. He continued his graduate studies there, receiving the Ph.D. degree in 1957 with a joint major in Veterinary Science and Zoology and a minor in Pathology. He did all this while still a bachelor. His life became a bit more exciting and complex when he came to New Jersey to embark on his professional career. He met a native New Jersey girl, Mary Mastin, whom he married in 1962. Their first daughter, Jenifer, was born while he was a visiting researcher at the University of Cambridge in England in the laboratory of Dr. Lawson Soulsby, whom many of you know. The second child, Peter, was born in New Jersey, and their second daughter, Betsy, was born in Australia during his stint at the Merck, Sharp & Dohme Veterinary Research & Development Laboratory "Down Under." Recently, Bill and Mary celebrated their silver wedding anniversary.

Bill's first position was as Research Associate at the Merck Institute of Therapeutic Research in 1957. Within a very short time, he progressed to Research Fellow, Senior Research Fellow, Director of Parasitology, and in 1976 to Senior Director of Basic Parasitology. Since 1984, he has been Senior Scientist and Director of Assay Research and Development. His professional and scholarly activities outside Merck also began to flourish. He was voted into many professional societies for which he served in many official capacities. He became the President of the New York Society of Tropical Medicine in 1970, the President of the New Jersey Society of Parasitologists in 1976, and the President of the International Commission on Trichinellosis in 1980. I, as editor of the *Proceedings of the VI International Conference on Trichinellosis,* had the pleasure of dedicating the *Proceedings* to him as President of the Commission. His affinity for the office of presidency was again evident when he became the President-Elect of the American Society of Parasitologists in 1985 and the President in 1986. In addition to serving as a key officer of these and other societies, his professional honors have included serving as a member of the Special Study Section of the NIH Tropical Medicine and Parasitology, the WHO Scientific Working Group, and of the editorial boards of the *Journal of Parasitology, Experimental Parasitology, Proceedings of the Helminthological Society of Washington,* and the *American Heartworm Society Bulletin.* He was an associate editor with me on four *Proceedings of the International Conference on Trichinellosis.* He is also an academician; he is presently Adjunct Professor of Parasitology at the New York Medical College and the University of Pennsylvania.

His invited lectures, seminars, and publications are too numerous to mention. However, I would like to briefly comment on his publications. His most significant contributions have been in the area of chemotherapy of parasitic infections but the following few publications exemplify his broad and varied research interests: (1) The nature and possible significance of the pigment in fascioloidiasis, (2) The efficacy of surface active agents in stimulating the evagination of cysticerci *in vitro,* (3) Mating success and fecundity of pairs of *Trichinella* larvae administered to mice, (4) Unimpaired infectivity of the nematode *Haemonchus contortus* after freezing for 44 weeks in the presence of liquid nitrogen, (5) Immunization of rats against *Ascaris suum* by means of nonpulmonary larval infections, (6) Can alcoholic beverages provide protection against trichinosis?, (7) History of trichinosis: Paget, Owen, and the discovery of *Trichinella spiralis,* and (8) Ivermectin: A potent new antiparasitic agent. Besides publications of research findings, he has edited two definitive reference books: (1) *Trichinella and Trichinosis,* and (2) *Chemotherapy of Parasitic Diseases.*

Already, it should be apparent to you that our President is a very versatile individual who believes in diversification. In addition to his scientific accomplishments, he has pursued and succeeded in a totally diverse area of creativity, the world of drama. His thespian talents have earned him accolades that are usually reserved for stars on Broadway. Time permits mentioning only two of his many triumphs, the role of Sidney Bruhl in the Summit Playhouse Association's production of "Deathtrap," and the role of the Old Actor in the New Jersey Public Theatre's production of "The Fantasticks." Let me quote one critic's review of his performance in "The Fantasticks": "The Old Actor (William Campbell) steals every scene he is in . . . ," and she ends the

* Presidential Address, 62nd Annual Meeting, American Society of Parasitologists, 5 August 1987, Lincoln, Nebraska.

review, "'The Fantasticks' continues at the N.J.P.T., at 118 South Ave., E., for three more weekends, until April 20. So who needs New York?"

Those of you who know Bill well have appreciated one of the most delightful characteristics of his personality, his keen sense of humor. At times, the subtlety of his humor has eluded me. I received a very cryptic note from him concerning an article entitled, "Mozart's Death Attributed to Parasitism" by C. A. M. Bell that appeared in *Parasitology Today*. The note read, "Charles, did you notice the latest paper on ivermectin and sheep disease?" I must admit that I had to reread the article to understand the note. Let me share with you the critical sentence in the article: "Since Sir Auptic Maynge was born and raised near Aberystwyth, it is perhaps fitting that the article will appear in a Festschrift issue to be published in honour of the Welsh composer Ivor Mekten." The note becomes meaningful if we read into the beginning and end of the sentence the words "psoroptic mange" and "ivermectin." I am sure that those of you who read that article in the April fool issue of *Parasitology Today* appreciated his creative talent as a humorous writer. He is also a poet as some of you may know if you read his "Vectorial Notes of a Restoration Poet," which appeared a year later in *Parasitology Today*.

Now that I have consumed a good portion of the time allotted for the Presidential Address, please allow me to present to you the multifaceted and multitalented President of our Society, Dr. William C. Campbell, who will speak on "Heather and Ice: An Excursion in Historical Parasitology."

HEATHER AND ICE: AN EXCURSION IN HISTORICAL PARASITOLOGY

ABSTRACT: Of those who have contributed to parasitology, two, A. E. Wilson and E. L. Atkinson, led lives of remarkable adventure and acquired fame beyond the realms of science. The lives of their fellow-adventurers are commemorated in the scientific names of more than a dozen helminth parasites.

In recent years I have written with perhaps excessive zeal about my primary interest in parasitology (chemotherapy) and about one of my avocations (trichinosis), so it is time for something completely different. The locale of this year's meeting is one of great historical significance in parasitology, and we are meeting jointly with the Wildlife Disease Association—so I have chosen a theme that is historical in nature and that deals in large measure with parasites of wildlife.

Back around the turn of the century the grouse population of the Scottish moorlands used to be periodically decimated by a mysterious disease. The human population was in consternation because the birds were dying before anybody had a chance to kill them. This upset everybody, not just the landed gentry and the shotgun-toting aristocracy, but also the beaters and ostlers, the bootblacks and the parlormaids—everybody whose livelihood was affected. Something had to be done; so in 1904 the local gentry formed a Committee of Inquiry on Grouse Disease. From the beginning its work had a parasitological motif because one could hardly ever open a grouse without finding a handful of tapeworms in it. Years before, old Spenser Cobbold had described a new species of nematode from the grouse and he thought that *that* was the cause of the disease; but lately it was said to be a bacterial pneumonia. Now the Grouse Committee, being composed of lay-men, was in no position to try and sort out these competing claims. Disease, whether of man or beast, was a medical problem—so they hired a young English physician to come up to Scotland to investigate the problem.

I want to tell you a bit about this man and about another young physician. They both took up parasitology; and by discussing them I hope we can get at least a glimpse of parasitology as it was in the first two decades of the present century. One could try to do the same thing by reviewing the literature of the time or by considering the really famous names in the field. But I want to do it by focusing on two young men who are really more representative in the sense that they contributed, but did not become "big names" in our field.

I also want to inject the human element further by taking a look at a few of the names of parasites. When we are dealing with one of the anisakines that cause disease in man, do we ever stop to think that it is named after this ship (Fig. 1)? When we are confronted with another, do we wonder why on earth it was once named after this young woman who had nothing whatever to do with parasitology (Fig. 2)?

To return, then, to our young man in Scotland: he was Edward Adrian Wilson—tall, angular, a convalescent from tuberculosis, but not at all frail (Fig. 3). He was an individual who must have been exasperatingly close to perfection. Written accounts of his life are filled with the most unqualified accolades—tributes to his physical, mental, and moral excellence—tributes that glow with a consistency that is positively

FIGURES 1, 2. **1.** The Terra Nova. **2.** Kathleen Bruce.

monotonous. I suppose that were he alive today, we could dig up some scuttlebutt about him—some nanogram of nastiness. But to judge by the record, Edward Wilson was unquestionably a hero among heros. He was a physician, but what he really excelled at was ornithology, and what he really, *really* excelled at was drawing and painting (Fig. 4), and by combining these two talents he had already made a reputation as a wildlife illustrator. But here he was tramping through the heather—he went everywhere, talked to every-

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FIGURES 3, 4. **3.** Edward Wilson. **4.** Drawing by Wilson.

one, he dissected thousands of grouse (including, by mistake, some that had been sent to him for his dinner), and he concluded that Cobbold had been right, that the disease was due to the nematode parasite, *Strongylus pergracilis* now called *Trichostrongylus tenuis*. But he really was not qualified to solve the problem on his own, so the Committee did two very smart things. First they called in Arthur Shipley of Cambridge, a distinguished parasitologist. (Incidentally, he is the one who was so impressed with the variety of ectoparasites on birds that he once remarked that birds are not so much birds as aviating zoological gardens.) Shipley discovered nematode larvae hanging from the dewdrops on the heather (Shipley, 1911). Grouse eat young shoots of heather, so this suggested a possible mode of infection. Wilson harvested eggs from infected grouse and collected larvae from cultures and tried to infect birds experimentally, but he had no success and so the Committee did their other smart thing; they called in Robert Leiper—Leiper of the London School. Leiper was then a young man in his twenties but already determined that, come what may, he would become a parasitological superstar—which he did (so he cannot be the other hero of our story). And he proceeded to do excellent work, collecting eggs and harvesting larvae, and studying their development, and showing that the first-stage larva had to develop and molt, and that the second-stage larva also molted but retained its cuticle as a sheath, and that the third-stage larva was in fact the infective stage (Leiper, 1911). Using this new knowledge, Leiper and Wilson succeeded in infecting grouse experimentally (Wilson, 1911). Now here we have the essence of the very important strongyle life cycle. The basic elements of that life cycle were pretty well known by the end of the first decade of the present century, but the details had to be worked out for every species and the work of Wilson and Leiper was an example of that.

When this work was just about finished, Wilson received a call to go to the Antarctic as Chief Scientific Officer on the expedition that Captain Robert Falcon Scott was leading to the South Pole. We cannot digress to ask why that should have come about, but Wilson actually was the one man that Captain Scott absolutely insisted on having with him on his attempt to be the first man to reach the South Pole. You may recall that Captain Scott and some of his companions died on that expedition. And that their bodies were subsequently found, and with their bodies the diaries and letters that contributed so much to making Scott's Expedition the most famous of all Polar expeditions. What you may not all realize is that the man who found the bodies and the letters was another physician–parasitologist. He was Edward Leicester Atkinson, a medical officer in the Royal Navy (Figs. 5–8). Unlike Wilson, he was short and stocky. Where Wilson was of artistic and philosophical bent, Atkinson was a man of action. He was in fact a Navy boxing champion; yet he was a very quiet, conscientious, and modest person.

The Expedition had to spend a winter in continuous darkness in Antarctica before they could make their assault on the South Pole and it was Atkinson's job, among other things, to examine the fish and marine mammals of the Antarctic coastline for parasites—and here we see him (Fig. 6) in his lab in the little hut in which the entire Expedition lived. In Figure 7 we see him catching fish in a trap on an occasion when the temperature was −40 F. The other man (on the left) happens to be the Expedition cook—who was presumably interested in the fish for a different but equally professional reason.

By coincidence Atkinson's parasitological training happened to have been done under none other than Robert Leiper at the London School of Tropical Medicine. Atkinson was thus pretty well versed in the subject and, as a result, that winter of waiting was enlivened by lectures on parasitology! Captain Scott himself learned a bit about hookworm disease, filariasis, dracunculosis, trichinosis, trypanosomiasis, and malaria, and the names of the parasites, in Scott's own phonetic spelling, may still be found in the pages of his diary in the British Library. Atkinson also made the surprising discovery that the sledge dogs on the Expedition had heartworm, *Dirofilaria immitis* (Atkinson, 1919). Some had pronounced hematuria and anemia, suggesting the life-threatening acute form of heartworm disease that we now call "caval syndrome." Quite a few of the dogs died, and Atkinson thought heartworm was the cause of death. It is believed by many that one of the reasons Scott and his companions died in Antarctica was because they did not make full use of the dogs that they took. Various explanations are given for this; but nobody has raised the possibility that Scott might have failed to use the dogs properly because the dogs were not fit—because they had heartworm. As a parasitologist I am tempted to argue that Scott was defeated

FIGURES 5–8. Surgeon-Lieutenant Leicester Atkinson. **5.** Portrait in uniform of Royal Navy. **6.** Helminthological work in the Expedition hut. **7.** Collecting fish for parasitological examination (with Clissold, the cook). **8.** One of Atkinson's frostbitten hands.

by a parasite. I suppose that would be going too far—but it just might be true!

Well with two physician–parasitologists on the Expedition we owe it to them to at least take a minute or two to see how they fared. Here is a parasitologist's map of the Expedition route (Fig. 9). From the bottom of the Beardmore Glacier the Expedition consisted of three sledge teams—groups of four or five men, hauling heavy sledges for 150 miles up the Glacier just to reach the Polar Plateau. Only one sledge team would make the assault on the Pole and of course everyone on the Expedition hoped to be picked for that final phase. After all, this was considered the last big geographical challenge on earth. Atkinson was the leader of one team and at the top of the Beardmore Glacier his team was sent back to Base. Scott did not give any reason, but we can guess. During the winter Atkinson had gone out of the hut to take a temperature reading, and in that dark and featureless landscape he had lost his bearings when only about 100 yards from the hut. When he stumbled back 5 hours later, he was mentally confused and suffering from frostbite (Fig. 8). Getting lost when you are almost on your own doorstep was not the sort of mistake that Captain Scott was likely to forgive. Later, the second team was sent back, leaving only one (Fig. 9). Of course our other physician–parasitologist, Edward Wilson, was a member of the team that remained—the final team. There was never any doubt that he would be chosen as one of those select few; he was simply that sort of person. In January 1912 they made it—a physician–parasitologist reached the South Pole (Fig. 9). Here is the famous group portrait (Fig. 10). The camera was operated by a string held by one of them and they are a rather sad group because

FIGURE 9. Map of Expedition route. Return of Atkinson was on 21 December 1911 at latitude 85° south. Wilson was selected for final team 3 January 1912 at 87° south; reached the South Pole 17 January 1912; died March 1912. Based on map by Apsley Cherry-Garrard.

they had found that Amundsen had beaten them to the Pole by just about a month. Wilson of course was even then incapable of despondency.

On the way back all five men died, and the map (Fig. 9) shows where Atkinson's search party found three of the bodies including the body of his fellow parasitologist and fellow physician, Edward Wilson.

When Atkinson got back to England he had worms, bottles and bottles of worms. He continued to be an officer in the Royal Navy, but he was posted to the London School in order to work on these worms together with Robert Leiper. They published descriptions of the worms in two papers, the first one (Leiper and Atkinson, 1914) giving brief synopses, without illustration,

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FIGURES 10–12. 10. [illegible] standing left to right, Oates, Scott, Evans; seated Bowers, Wilson. **11.** Edward and Oriana [illegible] sledge team, four men on skis and one (who took the photograph) who was harnessed on foot in the [illegible]

and the second (Leiper and Atkinson, 1915a) giving a more expansive account of their findings. The collection provided not only new records of known species but also many new species. Almost all of them were named after members of the Expedition (Table I). Time allows me to mention only some of them.

TABLE I. *New species named by Leiper and Atkinson (1914) in honor of members of the Terra Nova Expedition.*

Trematoda	Acanthocephala
Hemiurus oatesi	*Echinorhynchus campbelli*
Aponurus bowersi	*Echinorhynchus rennicki*
Lepodora garrardi	*Echinorhynchus debenhami*
Podocotyl pennelli	
Allocreadium fowleri	Nematoda
Cestoda	*Kathleena* scotti*
Oriana wilsoni*	*Terranova* antarctica*
Dibothriocephalus lashleyi	
Dibothriocephalus archeri	
Tetrabothrius wrighti	
Anthobothrium wyatti	
Tetrabothrius creani	
Tetrabothrius aichesoni	
Tetrabothrius catherinae	
Tetrabothrius priestleyi	
Tetrabothrius nelsoni	

* New genus.

The tapeworm *Oriana wilsoni* was named after Edward Wilson's wife, Oriana Wilson (Fig. 11), whom he had met while they were both doing missionary work in the London slums (naturally Wilson had done missionary work in the London slums). Actually they were both devoutly religious. Edward Wilson awaited his death in that tent in a mood that bordered on cheerfulness. Oriana Wilson lived for many years but, as far as I can gather by reading between the lines, she was not able to look back on that event with equal equanimity.

The trematodes, too, were given Expedition names. For example, *Aponurus bowersi* was named after Lieutenant Bowers, one of the three who died at the last encampment. Now one of the most remarkable things about Bowers was that he had accomplished that final march to the Pole without skis. Being a last-minute addition to the final Polar Party, he had left his skis back at one of the depots (Three Degree Depot). The other four members of his team had skis (Fig. 12). Bowers had to harness himself in the middle and do his best to keep up with the others. And this was the cause of my greatest embarrassment in the course of this work. I was in the British Museum (Natural History) in London, examining the notebooks in which Atkinson had kept records of all his dissections in the Antarctic. During a break I wandered into the Mineralogy Department, and my eye happened to catch one of those three-dimensional "diorama" displays in a glass case. It was a model of an Antarctic scene with Captain Scott's little party on the way back from the pole, when all five were still alive, and they were collecting rocks. That is why the exhibit was in the Mineralogy Department. It had five little doll-like figures standing in the snow among some rocky outcroppings. Well, in the exhibit, all five of the model figures were wearing skis; so when I got home I wrote to the Museum. Bending over backwards to be polite and diplomatic, I pointed out that while the exhibit was of geological rather than historical import, there still was the matter of too many skis. The Head of Petrology wrote back, expressing his delight that the mistake had been found after the exhibit had been on view for many decades. He had checked the matter with the experts of the Scott Polar Research Institute in Cambridge. They said I was right about the skis and so the skis would be removed from the figure of Bowers. Well just at that time I discovered to my horror that Bowers, on his way *back* from the Pole, had found his skis where he had left them—he would have had them when they stopped for the rocks. I quickly dashed off a letter, of suitably groveling kind, to the Head of Petrology. He, meanwhile, had added a note saying that they had removed the skis from Bowers. It had meant that they also had to change the stance of that figure from that of a skier to that of a walker, but they had managed to do it. Fortunately, the Head of the Petrology, Dr. Alan R. Woolley, proved to be a man of infinite patience and good humor.

Hemiurus oatesi was named after Captain Oates (Fig. 13), the most famous name of the expedition after Scott himself—because of his famous self sacrifice. Severely frostbitten, he could no longer keep up with the team and was jeopardizing their survival and so he walked out into the blizzard.

Podocotyl pennelli was named after Lieutenant Pennell, navigator of the Expedition ship. Later he became a commander on a much more imposing ship, the cruiser H.M.S. Queen Mary. At the battle of Jutland, during the first World War, it received five direct hits and some 1,300 men went to the bottom of the sea—including the man after whom *Podocotyl pennelli* was named.

Finally the nematodes: *Terranova antarctica,*

one of the anisakines, was named after the ship on which the Expedition sailed, the Terra Nova (Fig. 1). Some would prefer the name *Phocanema* for this worm, but we are not concerned here with the current validity of the names. *Kathleena scotti* was named after Kathleen Bruce, later Mrs. Scott, later Lady Scott, later Lady Kennet (Fig. 2). She was a sculptor, and as a young woman she was studying in Paris when she came to the conclusion that she would like to have a baby. In order to accomplish this she decided, rather unnecessarily one would think, to leave France. She further decided that even though she was an artist she would get married. Actually she did not just want a baby, she wanted a son—not just an ordinary everyday sort of son, but a heroic son. And so she was not merely interested in finding a husband *qua* husband, but more especially in finding a husband who would be qualified to be the father of her son. And so she gave up the gaiety of *la rive gauche,* put up her hair, and settled for the daily round of afternoon tea in Edwardian London. She met young Captain Scott and decided that he had the qualities she most admired; and so she married him and bore him a son. Like her late Queen before her, she found herself falling more and more in love with her husband. A few years later, she went all the way to New Zealand to meet the Terra Nova returning from Antarctica. Edward Wilson's wife was there too. But their husbands, as we know, were not on board. And, as it happened, it was the duty of our other parasitological hero Atkinson to escort the two women back to England, where Kathleen resumed her sculpting and mothering (Fig. 14).

That paper written by Leiper and Atkinson, with so many new species, is a paper that is widely cited because of technical taxonomic considerations. Atkinson and Leiper were subsequently to quarrel, and it has been suggested that the friction between them may have originated because Leiper (Fig. 15) made himself the senior author. I have some difficulties with that. In the first place, when one was working with Leiper, one did not *need* a basis for friction. Leiper be-

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FIGURES 13–15. **13.** Capt. Oates. **14.** Kathleen Scott née Bruce, and her son Peter (now Sir Peter). The inset, showing the Antarctic grave site, is part of a composite picture published in the 1930's by Brown Bros. of London. **15.** Robert Leiper.

lieved that a bit of friction made people work better and he made sure there was always lots of it around. Secondly, while Atkinson undoubtedly shouldered most of the microscopical labor, I think he would have recognized that Leiper's expertise in the subject matter was at least as great a contribution. And finally, if this had been a major source of friction, the thing that happened next would probably not have happened next.

At the beginning of 1914 the London School sent an expedition to China. It consisted of Leiper and one assistant; and the assistant he chose was Leicester Atkinson. They were going to China to study schistosomiasis. The Royal Navy said they were letting Atkinson go along because schistosomiasis was of concern to the crews of British ships on the Yangtze River. Well, perhaps. But the Colonial Office, which was paying the bill, was more interested in African schistosomiasis. Leiper, of course, wanted to solve the problem of schistosome transmission. Others, too, wanted to solve the problem of schistosome transmission; which raises the question: What was known at this time about the transmission of parasites by invertebrate vectors? By now (1914) the idea of *arthropod* vectors was no longer novel. By 1914 it was known that arthropod vectors were involved in the transmission of filariasis, babesiosis, trypanosomiasis, and malaria (and indeed plague, dengue, typhus). The role of *snails* in the transmission of trematodes was also known (the snail host of *Fasciola hepatica* had been known for years), yet in 1914 people were arguing about whether or not vectors were involved in schistosome transmission.

It was pretty well established that schistosomiasis was associated with exposure to water. But it was not known whether infection was due to miracidia, which could be seen to hatch from eggs, or to cercariae, which might emerge from some hypothetical snail host. Furthermore, regardless of what the infective stage was, it was not known whether it entered the human body through the mouth, or through the skin, or through the urogenital opening. The urogenital route seemed quite likely because of the blood in the urine of many patients in Africa. In fact that route seemed so probable that British troops going to Egypt were told that when bathing in rivers and streams they should wear condoms. (One can perhaps imagine an English soldier at home packing for foreign service and saying to his spouse "Those?—Oh, *those*! Those are just for swimming."

In Cairo the great parasitologist Arthur Loos said there was no snail host. Leiper, an old collaborator of Loos, was one of many people trying to prove him wrong. Leiper knew that *Schistosoma japonicum* infects many animals besides man, so he thought that it would be easier to do the job of finding a vector in the Far East rather than Africa. *That* is why he set off to China. Well Leiper and Atkinson spent several months in China and they could not even get a stool specimen from a single infected patient. Professor Nelson, who has written about this episode (Nelson, 1977), points out that neither of them had sufficient diplomatic skills. We need to remember that China had been a republic for only 2 years and that the Chinese had for some time been very fed up with Europeans, especially British. Now here were two very proper British gentlemen trying to persuade Chinese people to hand over their feces. They never got any. They finally got an infected dog and got some miracidia but their attempts to infect snails were unsuccessful. By this time the two men were really getting on each other's nerves. In the archives of the Scott Polar Research Institute in Cambridge there are several letters that Atkinson wrote at this time. He said very clearly that he did not consider Leiper a man to be trusted, and in one letter Atkinson said that he should give Leiper a sound thrashing for the good of his soul. However, before the men actually came to blows World War I broke out and Atkinson hurried home.

The Gallipoli campaign was one of the War's most pathetic fiascos. The problem was not just the Turkish soldiers up on the cliff tops or the Young Turks up in Constantinople. The problem was also disease. The problem was dysentery and typhoid. And the problem was flies. The Royal Navy, having landed men and guns on the peninsula decided to tackle the sanitation, and so they landed Leicester Atkinson. The troops in this little corner of the Gallipoli peninsula were sandwiched between the beach and the front line. On the beach stood long lines of cavalry horses; and where you have cavalry *horses* you have cavalry *horse manure*. When the wind blew from the South it brought in clouds of filth flies (*Musca* and *Fannia* species) and when the wind blew from the North it brought blowflies (*Calliphora* and *Lucilia*), which were breeding in the decay-

ing flesh that was abundantly available at the front line. What did one have for fly control in those days? Well, one had chlorinated lime to put on top of the heaps of accumulated human wastes and that was about all. Atkinson did experiment with a new insecticidal mixture called Mixture "C." He sneaked out into no-man's land and tried it on the corpse of a Turkish soldier. It was a great success, although the trial, being within sight of enemy snipers, was of limited experimental design.

In Gallipoli, Atkinson also had an opportunity to look into protozoal infections as the cause of human disease. For the most part he just made incidental clinical observations, but they broadened his scope as a parasitologist, and give us an opportunity to recall that it was already known at the time of World War I that there was bacillary dysentery and amebic dysentery. It was even known that *Entamoeba* in man was of two kinds, pathogenic and nonpathogenic, and they had been given the names by which we still know them.

After Gallipoli, Atkinson was posted to a naval hospital on the South Coast of England where he had an opportunity to write up his experiences in Gallipoli and his prior experience in China (Leiper and Atkinson, 1915b; Atkinson, 1916a, 1916b). The trouble with that was that it brought up the subject of Leiper again. Leiper, naturally, had been busy since we last left him. What he tended to be very quiet about was that just *before* he and Atkinson had gone to China, Leiper had learned that the Japanese claimed to have found a snail host for *Schistosoma japonicum*. So before Leiper left China he made two short visits to Japan and found that Miyairi and Suzuki had indeed documented the molluscan transmission in almost complete detail. With the help of Fujinami and other Japanese workers Leiper was able to confirm the transmission of *S. japonicum*—by establishing an infection in a single mouse. Now with the outbreak of war, Leiper got himself posted to Egypt which was just where he wanted to be to find the snail hosts of the other schistosome species. And that was just where his rival Loos was—his main opponent in the schistosome controversy. But Loos was a German citizen and had to vanish from Egypt immediately (turning up later in the German army). He left his lab and library sitting empty—just right for Leiper to move into. Then the wounded soldiers and dysentery cases started pouring into Cairo from Gallipoli. Lieutenant Colonel Leiper was asked to leave to make room for them. At first he refused (and later bragged about it), but eventually he had to go home—having already settled the matter of the snail hosts of the major schistosome species.

Atkinson finally persuaded the Navy to send him to France—to the Western Front. This was where he had always wanted to be. Now he was there with the big artillery guns of the Royal Marines. He was there with the mud and the wounded. Young men were pouring out of trenches into barbed wire under a hail of machine gun bullets. They were being blown up. They were being gassed. They were dying not by the thousand, but by the million—they were dying like flies—like flies sprayed with Mixture "C." The problem was not just bullets and mud, but also disease—of which a major component was ectoparasitic infestation. The war on the Western Front, after all, was being fought by hordes of lousy soldiers. Not only was lousiness in itself a bad thing, but it was responsible for the widespread occurrence of the louse-borne, typhus-like disease, trench fever. Atkinson worked under these conditions for a year. But the Germans had big guns too and one of them blew up Atkinson's hut. He was severely wounded in the face and eyes but he refused to go home. He was patched up, got wounded again, and carried on coping with his wounds as well as his patients for another whole year on the Western Front. And then he landed back at the same naval hospital in the South of England, but this time as a patient. The surgeons took the shrapnel out of his eyes—well they took most of it out—and they gave him a medal for heroism; but they would not allow him to go back to France. He had to settle for a job as a ship's doctor. As it happened, the ship was carrying explosives and his new assignment ended with a bang! When he regained consciousness immediately following the explosion he engaged in feats of heroism that you would hardly believe even if I had time to tell you. He was later found on deck, unconscious again, and was almost given up for dead. Back he went to the naval hospital—where they gave him another medal and took away one of his eyes. They patched him up again, and this time they allowed him to go back to France. Perhaps they knew what they were doing, because by the time he arrived in France, the guns were silent.

The Great War was a hard act to follow. Our man Atkinson was lucky. He was young. He had

his medical profession. He had his Navy career. He still had one of his eyes and all of his arms and legs; but his face was so patched up he was hardly recognizable, even to his friends. He was several times a hero; but the streets were littered with heroes. He found things to do, alright—he even won another medal! But there we must leave Leicester Atkinson except to note that a mere 14 years later, when he was retired from the Navy as medically unfit, recently married, recently a widower, depressed, and bothered by old head wounds, he died rather suddenly at sea. He was 46 years old. Over the circumstances of his death, the Royal Navy has drawn a veil. I tried to lift a corner of that veil but the keepers of Her Majesty' secret books very properly stuck to their guns. They told me I could see that particular secret book in the year 2004. I told them I expected to be a good deal less interested in the year 2004.

Does it matter that Wilson and Atkinson were the ones who did the work that they did? Does it matter why a worm was named after a ship? Does it matter why a worm was named after a certain young widow? Perhaps not. It is of course the scientific facts that count. But I like to believe that the richness of our discipline depends not only on the cold facts but also on the human fancies and foibles that surround them.

ACKNOWLEDGMENTS

I am most grateful for the gracious reception and willing assistance received from the librarians and archivists of The Scott Polar Research Institute, Cambridge, England, The British Library, London, England, and The Alexander Turnbull Library, Wellington, New Zealand.

Author's note: Only minimal documentation has been added to the text of the talk as delivered. It is intended that more detailed accounts of some of these episodes, with full documentation, will be published elsewhere.

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INTRODUCTION OF DR. BRUCE MARTIN CHRISTENSEN AS THE HENRY BALDWIN WARD MEDALIST FOR 1987

Walter M. Kemp
Department of Biology, Texas A&M University, College Station, Texas 77843

The Henry Baldwin Ward Medal represents the highest recognition of professional accomplishment awarded by the American Society of Parasitologists. The recipient must have achieved a position of leadership in some aspect of parasitology by self-directed research, excellence in teaching, and in professional service prior to the age of 40. The recipient must have been a member of the Society for at least 3 years prior to nomination. To receive the Ward Medal is a profound professional acknowledgment for the Medalist, but in choosing our best members for the honor, we, as a Society, also honor ourselves. The value of the H. B. Ward Medal is most obviously ascertained by the quality of the recipient. Less obvious, but no less indicative, the Medal's value is defined by those who are qualified and fully deserving of the Medal, but for whatever reason never receive it. Our committee examined the credentials of five truly outstanding candidates this year. When faced with such intellectual talent and when evaluating such valuable contributions to our field, one is tempted mightily to attempt to award more than one Medal. I sincerely hope that in the future, each of you has the opportunity, or curse, of serving on the Awards Committee.

It is my honor and my privilege tonight to introduce to you the winner of the 1987 Henry Baldwin Ward Medal, Dr. Bruce Martin Christensen. Dr. Christensen was born on 16 November 1947 in Shell Lake, Wisconsin. He received the Bachelor of Science Degree in 1970 with a major in Biology and a minor in Chemistry from the University of Wisconsin at River Falls, and the Doctor of Philosophy in 1977 from Iowa State University, majoring in Entomology (with emphasis in Medical Entomology) and in Zoology (with emphasis in Parasitology). Between those degrees he served as a biological scientist in the United States Army, and those who know him well assure me that we have Dr. Christensen to thank for the Viet Nam War lasting so long. His doctoral dissertation was entitled "Biology of *Dirofilaria immitis* (Leidy, 1950) (Nematoda: Onchocercidae) and *Aedes trivittatus* (Coquillett 1902) (Diptera: Culicidae) in central Iowa," a topic which has retained considerable interest to Dr. Christensen. In terms of honors, he received the Clyde Black and Son Excellence Award for the outstanding Ph.D. student in Entomology at Iowa State in 1977, the H. W. Manter Award from the Annual Midwestern Conference of Parasitologists in 1976, and the Pound Research Award from the College of Agriculture and Life Sciences of the University of Wisconsin in 1986. He is currently serving on the editorial boards of the *Journal of Parasitology*, the *Journal of Wildlife Diseases*, and is an immediate past board member of the *American Journal of Veterinary Research*. He also is currently serving time as a member of the Tropical Medicine & Parasitology Study Section and the Parasitic Diseases Panel of the U.S.–Japan Cooperative Medical Sciences Program of the National Institutes of Health. Dr. Christensen holds membership in nine professional societies.

Academically, Dr. Christensen was a Temporary Assistant Professor of Zoology at Iowa State from 1977 to 1978, and as an Assistant Professor of Biology at Murray State University from 1978 to 1982 prior to his appointment at the University of Wisconsin–Madison where he is presently an Associate Professor of Veterinary Science. He lists over 50 publications in refereed journals. His laboratory is well funded by a surprisingly diverse set of funding agencies ranging from the NIH to the National Wild Turkey Federation. This latter agency particularly caught my eye because the research supported by it has resulted in a paper which is currently in press in the *Journal of Wildlife Diseases* entitled "Haematozoon Parasites of Rio Grande Wild Turkeys from South Texas." I take off my hat to anyone who is willing to protect the bourbon drinkers of Texas from haematozoon parasites.

Scientifically, Dr. Christensen's productivity has been most impressive. Four of the 10 years since the receipt of his doctorate, he was an Assistant Professor at an institution very long on teaching responsibilities and short on research environment. At that time he was the first recipient of an NIH grant ever awarded an individual at that institution, and he alone, and in collaboration with students and colleagues, published a number of classical entomological and parasitological surveys, all the while continuing his work on mosquito–filaria interactions.

Since his move to the University of Wisconsin, Dr. Christensen's work on mosquito immune responses to filarial infection has flourished. His current work literally defines the state-of-the-art in invertebrate immunology both in the outcome of investigation and in development of microtechniques with which to study biochemical and immunological phenomena in mosquitoes. Using the *Aedes–Brugia pahangi* models in addition to the *D. immitis* model, Dr. Christensen has clarified the role of the microfilarial sheath of *Brugia* in midgut penetration and immune evasion and has demonstrated parasite-induced suppression of the mosquito immune response. Dr. Christensen's work on the melanization response has significantly affected general concepts of invertebrate immune mechanisms, demonstrating a strong cell-mediated component to the melanization response and significant hemocyte proliferation and surface differentiation in response to filarial worm infection. He has developed microtechniques for studies of catecholamine levels and monophenol oxidase activities in *Aedes*. He currently is using these techniques to document changes in catecholamine metabolism during immune responses and to examine possible correlates between *Anopheles gambiae* strains susceptible and refractory to infection with *Plasmodium berghei*. He is also seeking support for

studies of the immune response to parasitoid infections in *Drosophila* mutants with known melanization lesions as a route to the definition of the biochemical mechanisms of insect immune responses.

Perhaps most illuminating of all are the comments of Dr. Christensen's colleagues and the following are representative quotes:

"In addition to Bruce's classical training which has allowed him to 'think like a parasite,' he is always willing to learn and apply contemporary technologies to his research. This general approach is always dispatched with sincerity, honesty, discipline, and, importantly, a good sense of humor."

"The program Bruce has built is outstanding. It is the result of the integration by Bruce of hard work, his own inquiring and creative mind, plus a broad variety of techniques and model systems derived from his dual experiences and education in modern experimental entomology from Wayne Rowley and from a broadly based curriculum in parasitology provided by Martin Ulmer at Iowa State. This unusual combination of view point, technical skills, and intellect have produced a unique series of studies which bring us closer and closer to a complete understanding of the potentialities of the insect's cellular immune system. Many of his published works are considered benchmarks by those in vector biology. His international recognition from these works continues to grow and is evidenced by his recent invitation to participate in the U.S.–Japan Cooperative Medical Science Program and to present a keynote address at the London Zoological Society."

The Chairman of the U.S.–Japan Cooperative Medical Sciences Program Panel in Parasitic Diseases (himself a Ward Medalist) commented, "During our recent visit to Japan Bruce again demonstrated his versatility, interactive capabilities, and the caliber of his research. He has proven to be a valuable member of the panel."

In regard to teaching, his Department Chairman wrote "In addition to these research accomplishments, Professor Christensen has developed an outstanding teaching program since his arrival on campus in 1982. He has revised our old veterinary parasitology from a didactic survey course to a comprehensive offering on parasite–host interactions. He is an integral member of a vital and growing parasitology group on campus, and is a good academic citizen—always willing to serve in any capacity to benefit the department."

"In summary, I believe Bruce's record speaks for itself. The diversity of parasites studied (from head lice to turtle protozoans) and techniques employed is indicative of a versatile and powerful intellect. The depth of his understanding of filarial worms and mosquitoes and their interactions is in my view unrivaled. Anyone who knows Bruce personally would affirm that his appetite for work and love of research have not dimmed one iota over the years. The probability of Bruce remaining productive and innovative is therefore very high, and I conclude his selection as a Ward Medalist would reflect well on our Society for many years to come."

Ladies and gentlemen, the Henry Baldwin Ward Medalist for 1987, Dr. Bruce Martin Christensen.

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HENRY BALDWIN WARD MEDAL ACCEPTANCE SPEECH

Bruce M. Christensen
Department of Veterinary Science, University of Wisconsin–Madison, Madison, Wisconsin 53706

Dr. Kemp, President Campbell, Secretary Schmidt, fellow members of the American Society of Parasitologists, and guests: I am deeply honored and humbled to be the recipient of this award. I am honored to know that my peers consider me worthy and humbled when I read the list of past recipients. To say that I am also happy is a major understatement. Upon receiving notification of my selection, it took five days, plus the realization that I had seven grant proposals to review, to bring me back somewhere close to earth.

Being selected to receive the Henry Baldwin Ward Medal requires the good fortune of having colleagues that think highly enough of your work to unselfishly put forth the effort required for the nomination, and it requires that the Awards Committee likewise appreciates the research area and accomplishments of the nominee. To get to a position where these events have a decent probability of happening usually requires some good fortunes, and a bit of luck, during the professional developmental period of the recipient. This has indeed been the case for me.

I was recruited out of high school by the University of Wisconsin at Madison and at River Falls by their respective wrestling coaches in 1966. As a high school graduate in a class of 53, I chose River Falls over Madison based mainly on my fear of the big city and big university. Choosing a university for the wrong reason enabled me to meet Dr. Robert Calentine while taking his course in invertebrate zoology. His enthusiasm for helminthology was contagious, and his willingness to accept me as an undergraduate research assistant had a significant impact on my life and future education. Working in Bob's laboratory for three years removed the mystique I had about scientists and research. I learned that most good scientists do not sit in their laboratories formulating and contemplating great thoughts, but rather have a love for their subject material, a sound reasoning ability regarding their observations, the willingness to repeat experiments until they are sure of their observations, and most of all, they have common sense. After publishing my first paper with Bob in 1970 on the specificity of caryophyllaeid cestodes for their oligochaete intermediate hosts, I was hooked on parasitology and on invertebrate host–parasite relationships. My association with Bob since my undergraduate days has progressed from that of advisor–student, to colleague, grouse-hunting companion, and friend.

Graduation in 1970, together with a draft lottery number of 107, provided an opportunity to experience a tour of duty in the United States Army. Advice from a benevolent recruiter and a great deal of luck enabled me to spend 30 months as a biological research assistant with the Medical Research and Nutrition Laboratory at Fitzsimmon's Army Medical Center in Denver. Studies on the effect high altitude has on physiological and biochemical functions in mammals took me to the top of Pikes Peak to live for three summers, and our studies resulted in two publications. This experience convinced me that biochemistry was basically "cookbook" research. In the last several years, Jim Tracy has shattered that concept and embarrassed me on several occasions regarding my biochemistry expertise.

Working in a federal research laboratory reassured me that a university environment was where I was most happy, and I was elated when Dr. Martin Ulmer offered me a teaching assistantship and laboratory space to conduct my doctoral studies in helminthology at Iowa State University. I arrived with a commitment to study caryophyllidean cestodes and tubificid annelids, but I registered too late for a physiology course and had to take medical entomology from Dr. Wayne Rowley during my first quarter. Over the next year my interest in mosquito-borne diseases reached such a high level that both Drs. Ulmer and Rowley encouraged me to double major in parasitology and medical entomology. Their willingness to function as co-major professors created an ideal environment for my graduate training. Resulting studies on the biology of *Aedes trivittatus–Dirofilaria immitis* associations occupied the majority of my time and formed the basis for my dissertation. Martin Ulmer and Wayne Rowley are very special to me. Martin Ulmer provided me with a solid, and essential, background in classical parasitology, and at the same time set the standard by which one measures an academician, scholar, and gentleman. He also gave me an appreciation for vodka martinis, premixed, stored in the freezer, and always available. Wayne Rowley taught me experimental biological research, and in-

stilled in me self-confidence and, at the same time, the necessity of self-criticism. I cannot emphasize enough the gratitude I feel for the influence he had on my development as a scientist. He is also one of the few major professors I know who was willing to spend an entire Sunday helping a graduate student manually copulate mosquitoes.

While at Iowa State I was fortunate to have the opportunity of working next to and with a number of fellow graduate students in parasitology and medical entomology. I profited from my association with all of them. Darwin Wittrock, in particular, was an outstanding source of intellectual stimulation, friendship, and good times. This has not changed in the last 14 years. Eain Cornford, an Ulmerite who preceded me at Iowa State, is an equally valued colleague. We tolerate each other's presumed great thoughts when everyone else has gone to bed. I am convinced New Zealanders do not require sleep. Sam Loker, one of the best snail biologists, parasitologists, and comparative immunologists I know, is one of those rare colleagues and friends that makes one a better scientist and person simply by association. My professional interaction with Sam has grown stronger since our graduate student days, as has our friendship.

In 1978 I received an offer from the Biology Department at Murray State University in Kentucky and I joined that faculty in August. I immediately wrote my first NIH grant proposal dealing with the immune response of mosquitoes against filarioid nematodes. I was pleasantly surprised and the university was shocked when the proposal received a fundable score. Some details had to be worked out rapidly, such as the establishment of an animal care committee and figuring out what cost sharing was all about, but the university and I learned the ropes together and the funding actually began. Maintaining an active research program was at times difficult during my four years at Murray State. Teaching loads, by necessity, were heavy, and equipment and areas of expertise were limited. But thanks to an understanding chairman and the presence of enthusiastic students, we conducted some good science. A special thanks to two of my fellow faculty members at Murray State, Arthur Williams and Joe King. They understood my enthusiasm for research without knowing parasitology, and at least feigned interest in my excitement over something new or unexpected. They picked up my spirits whenever they needed a boost.

When the Department of Veterinary Science at the University of Wisconsin invited me to interview for an open parasitology position, I was excited about the possibilities, but also aware that a parasitologist working with an invertebrate host model might not fit into the scheme of things in Veterinary Science. The Department's decision to hire me was a major turning point for my research program. I arrived at UW at nearly the same time as John Oaks, and in the ensuing few years, Jim Tracy, Bob Grieve, John Mansfield, and Don Wassom joined the faculty. This is as fine a group of parasitologists as I could ever hope to be associated with, and together we have established the Center for Research and Training in Parasitic Diseases. The establishment of this Center at UW enhances our research and training programs and provides additional incentive for the entire group.

I consider myself a parasitologist and medical entomologist in the broadest definitions of these titles, and I feel fortunate to have received training in both classical and experimental parasitology. My earlier years probably emphasized classical aspects for the most part, but I have been drawn very rapidly over the years into experimental biochemistry and cellular and molecular biology. I have not been dragged into these areas screaming and kicking, but have made this transition willingly. It was essential for me to do this if I wanted to answer some basic questions concerning mosquito–parasite relationships. Speaking to you this evening provides an opportunity to register my dismay over the arguments I hear at every meeting regarding the relevant merits of "classical" versus "modern" parasitology. To prefer one area over the other is a matter of interest and preference, but to consider one area more deserving or important is simply intellectual laziness.

Whatever reputation I might have as a parasitologist is undoubtedly based on my work with mosquito-filarial worm relationships, and these studies continue to constitute the majority of my laboratory's efforts. I emphasize laboratory's, because it has been the combined efforts of graduate and undergraduate students, postdoctoral associates, technicians, visiting professors, and fellow faculty members that have been responsible for whatever success my research program has experienced. Through this team effort I believe we have made reasonable progress in our attempts to unravel the complex relationships that exist between mosquitoes and the filarial worms they transmit. But we still do not know very much.

Although there are differences in the immunological capabilities of different species and strains of mosquitoes, it has become apparent that even mosquitoes highly susceptible to filarial worm infection possess an inherent ability to encapsulate and destroy microfilariae. However, passage of *Brugia* microfilariae through the midgut of susceptible mosquitoes allows them to avoid immune recognition. Surface changes on microfilariae are associated with midgut penetration, but we have yet to determine how these changes may relate to immune evasion mechanisms. Once microfilariae enter the indirect flight muscles and begin developing as first-stage larvae, they actively suppress the immune capabilities of the vector. It is apparent that the parasite's response to the host, as well as the host's response to the parasite, determines whether the vector–parasite relationship will be compatible. Undoubtedly, the most exciting research area involves the clarification of those mechanisms that allow a filarial worm to develop in a biochemically hostile environment, but we realized very quickly that it is difficult to assess how the immune response of a mosquito is perturbed by a parasite when we know very little about the mechanisms of the response itself. Consequently, we are now concentrating on studies designed to clarify the cellular and humoral components of mosquito immune responses, and the biochemical mechanisms functioning in the production of protein–polyphenol complexes necessary for parasite destruction.

I have been fortunate to have Toni Nappi actively collaborating with my laboratory's program. Toni is the most knowledgeable insect immunologist, creative thinker, and energetic scientist I have ever met. His

enthusiasm for research can best be compared with pouring warm coke over ice, uncontrollable effervescence. He takes his science seriously without taking himself seriously. When I asked him for suggestions regarding my acceptance speech, he said, "Don't be too humble, you're not that good!" Toni is a very close friend, as well as a colleague, and deserves a good share of the credit for my laboratory's past and future successes.

Jim Tracy is an outstanding biochemist, who has made significant contributions to our research efforts during the last several years. I would like to believe that it is my influence that is enabling Jim to also become an outstanding parasitologist, but all I can be sure of is that my efforts in this regard have been more successful than Jim's efforts to create another biochemist.

Because of my emphasis on studies of the defense response of mosquitoes to parasites, I am considered a member of the small but growing ranks of comparative or invertebrate immunologists. Compared to vertebrate immunologists, our Society does not harbor many members who study such hosts as snails, amphipods, or mosquitoes and the mechanisms whereby they respond against invasion by parasites, or the means by which the parasite avoids immune destruction within these hosts. Although parasitologists have always been interested in which species may or may not function as an intermediate host for a particular parasite, little emphasis has been placed on understanding the mechanisms responsible for this susceptibility and refractoriness. The importance of clarifying these mechanisms to our understanding of the epidemiology of parasitic diseases is obvious to individuals working in this area. Standing here this evening provides verification that our Society also recognizes this area of research as important and meaningful.

Before closing, I must acknowledge those individuals, that I have yet to mention, who have helped me along the way. Betty June Myers provided the guidance and patience so helpful to a young scientist writing his first NIH grant proposal. Mary Lou Pritchard and Robin Overstreet spent too many hours without any reward explaining the do's and don'ts involved in writing taxonomy papers. Bob Lewis, one of the finest systematic entomologists I know, set an example of an academician and provided verification that one need not compromise in their beliefs. Dan Colley and Harley Sheffield have demonstrated their belief in my ability and provided me with unique opportunities few parasitologists get to experience. Larry Ash has encouraged and supported my research. Encouragement from an established authority means so much to a young investigator. Our society benefits from having members as nice as Larry Ash. All of my past and present students, who have done the majority of the teaching and have given me valuable lessons on faculty–student relationships as well as parasite–host relationships. My wife, Linda, has functioned in a much greater role than simply furnishing moral support and understanding. She has been a valuable part of my research effort. She is a trained biologist who reared and dissected mosquitoes at Murray State University when I had no funding for technical support. She continues to be the organizer and stabilizing force in my laboratory where students and staff are clamoring for space and material. My son, Brett, was a constant laboratory and field companion during my graduate student days and he continues to be a companion and friend, as well as a son. He has made it extremely easy to be a parent. A sincere thanks to so many of you in our Society who have shared your thoughts with me and listened to mine. You are the ones that make this Society so very special for me.

I do not work for a living. I work on committees at times, and I do consider grant writing work, but teaching and research are enjoyable pastimes that do not fit my definition of work. I am receiving this award tonight basically for having fun, and because I have benefited from some accidental events, good fortune, and an association with many good people. But I will take some of the credit for the accomplishments on which this award is based. Ladies and gentlemen, thank you again for this wonderful honor.

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AMERICAN SOCIETY OF PARASITOLOGISTS

Sixty-second Annual Business Meeting
5 August 1987
Business Administration Auditorium,
University of Nebraska, Lincoln, Nebraska

The Sixty-second Business Meeting was called to order by President Campbell at 1:30 P.M. in the College of Business Administration Auditorium at the University of Nebraska, Lincoln, Nebraska, on 5 August 1987.

President Campbell welcomed the members and guests present and called for reports of officers. Secretary-Treasurer Schmidt, Co-Editors Mettrick and Desser, and Program Officer Mead reported on the activities of their offices. These and other reports are presented in detail in the minutes of the Seventy-seventh Council Meeting.

President Campbell called on Secretary-Treasurer Schmidt to report to the membership the significant actions of the Seventy-seventh Council Meeting, which was held 2 August 1987. These actions are noted in the minutes of the Council Meeting.

President Campbell called for reports of the following committees:

1. *Tellers* (T. J. Nolan, Chairman; L. S. Blair and R. Wendt)

Secretary-Treasurer Schmidt reported the results of the 1987 election as presented in the minutes of the Seventy-seventh Council Meeting.

2. *Auditing Committee* (K. R. Kazacos and C. M. Bartlett)

"Mr. President and fellow members of the Society, we have examined the petty cash accounts of the American Society of Parasitologists, and have found them to be in order."

The report was ordered placed on file.

3. *In Memoriam Committee* (J. L. Bennett, Chairman; R. Boisvenue and C. E. Tanner) Secretary-Treasurer Schmidt read the report as follows:

"Mr. President, we report with regret the deaths of the following members:

DR. CECIL A. HOARE, date unknown;
DR. WILLIAM B. LeFLORE, on 6 December 1986;
DR. CORNELIUS B. PHILIP, on 8 January 1987;
DR. JOSEPH E. SEMRAD, date unknown.

We request a moment of standing silence in memory of our departed colleagues."

The report was ordered placed on file.

4. *Resolutions Committee* (L. Margolis and S. A. Ewing)

"Mr. President, Members of the Society, and Guests:

Be it resolved that the American Society of Parasitologists expresses sincere thanks to:

—members of the *Local Arrangements Committee* led by Chairman John Janovy, Jr., for their excellent planning and hospitality;
—the College of Biological Sciences, University of Nebraska, for facilities, equipment, and financial support;
—the Nebraska State Museum, site of the warm and generous reception;
—Mary Lou Pritchard for providing tours of the Manter Laboratory;
—Norden Laboratories of Lincoln, Nebraska, for financial support;
—the *Registration Committee* under the direction of Larry Hammer;
—the Program Officer, Robert W. Mead;
—the Wildlife Disease Association for cooperation with program arrangements;
—and, finally, the students of the University of Nebraska who, among other things, supplied the audiovisual services, provided a superb party, and took care of the parking tickets."

The report was ordered placed on file.

5. *Honorary and Emeritus Members Committee* (J. Janovy, Jr., Chairman; R. Fayer and A. J. MacInnis)

J. Janovy, Jr., with approval of Council, nominated Dr. O. N. Bauer, Soviet Union, to fill a vacancy in our list of Honorary Members. The nomination was seconded and unanimously approved by the quorum present.

No Old Business items were discussed.

Under New Business, the following items were discussed:

1. The results of the student paper competition were presented by B. Fried, Chairman, Special Student Awards Committee. The winners of this competition are:

   Glenn E. Kietzmann, Jr. of Iowa State University, Ames, Iowa for paper #31 entitled "Early Event of Caseous Nodule Formation in Ring Doves (*Streptopelia risoria*) Following Infection with *Trichomonas gallinae*," and

   Michael R. Lappin of the Departments of Small Animal Medicine and Parasitology, the University of Georgia, Athens for paper #32 entitled "Diagnosis of Recent *Toxoplasma gondii* Infection in Cats by Enzyme-Linked Immunosorbent Assay for Immunoglobulin M."

2. Brent B. Nickol announced that Scott L. Gardner is ASP's 1987 nominee for the J. Roger Porter Award, given by the U.S. Federation of Culture Collections.

3. The Special Student Awards Committee's change in status from *ad hoc* to standing, approved by Council, was discussed. It was decided that this question would go out on a mail ballot to the membership, or be on the agenda for the 1988 Business Meeting, to be voted on if a quorum is present.

President Campbell thanked the Society members for their support and expressed his appreciation for the work of the Local Committee, Officers, and Council Members-at-Large. He then turned the gavel over to President-Elect Castro.

President-Elect Castro declared the Sixty-second Annual Business Meeting adjourned at 2:30 P.M.

Respectfully submitted,
Gerald D. Schmidt
Secretary-Treasurer

J. Parasit., 74(1), 1988, pp. 20–27

AMERICAN SOCIETY OF PARASITOLOGISTS

Seventy-seventh Council Meeting
2 August 1987
Nebraska Union, University of Nebraska, Lincoln, Nebraska

The Seventh-seventh Council Meeting convened at 9:00 A.M. in the Nebraska Union Georgian Suites at the University of Nebraska, on 2 August 1987. Attending were President William C. Campbell, President-Elect Gilbert A. Castro, Vice President J. Ralph Lichtenfels, Immediate Past President Raymond T. Damian, Secretary-Treasurer Gerald D. Schmidt, Co-Editors David F. Mettrick and Sherwin S. Desser, Program Officer Robert W. Mead, Archivist Mary Lou Hanson Pritchard, and Council Members-at-Large Burton J. Bogitsh, Gerald W. Esch, J. K. Frenkel, W. Michael Kemp, and Raymond E. Kuhn. Also attending were W. Michael Kemp, President-Elect 1988 and Lillian F. Mayberry, Secretary-Treasurer 1988–1990; Committee Chairmen Gerald D. Esch, Stephen G. Kayes, Raymond E. Kuhn, and Jane A. Starling; and Affiliate Society Representatives John R. Bristol, Mary E. Doscher, and Willis A. Reid, Jr.

President Campbell called the meeting to order and welcomed those present.

The minutes of the Seventh-sixth Council Meeting were accepted as published in Volume 73, Number 1, of *The Journal of Parasitology*.

Leo Margolis and S. A. Ewing were appointed by President Campbell to act as Resolutions Committee, and the President appointed Kevin R. Kazacos and C. M. Bartlett to the Auditing Committee. The President recognized two new Council Members-at-Large for 1988–1991, Larry R. Ash and Jeff F. Williams, and expressed appreciation to outgoing Council Members-at-Large Gerald W. Esch and Jacob K. Frenkel. President Campbell also recognized Lillian F. Mayberry, Secretary-Treasurer for 1988–1990.

I. OFFICERS' REPORTS

A. President (W. C. Campbell)

It is a great privilege to serve as President of the American Society of Parasitologists and I am deeply grateful for the opportunity to do so.

I would like to express my thanks to all of the officers, members of council, and committee members who have provided unstinting support. In many cases the interaction between these individuals and the President is indirect, though nonetheless real. There are others with whom I have worked directly, and I would especially like to thank Dr. Lichtenfels, Dr. Schmidt, and Dr. Mead for making my job much easier than it might have been. We will have a formal resolution of thanks to our Local Committee, but I would add my personal thanks to Dr. Janovy and his associates who have made us so welcome in Lincoln.

Addendum: At the Annual Banquet on 5 August, I pointed out that this was the last year in which we would have the benefit of Dr. Robert W. Mead's talents as Program Chairman. He has served two full terms of 3 years each. It is a difficult job and Dr. Mead has done it extremely well. The thanks and best wishes of the Society were expressed by acclamation.

At the Banquet I also pointed out that in any society it is the Secretary-Treasurer who bears the brunt of the work and the stresses and strains associated with the organization. Dr. Gerald D. Schmidt has served for 7 years (more than two full terms) as our Secretary-Treasurer and is now relinquishing his duties. It is important to remember that Dr. Schmidt took office at a time when the business affairs of our Society were in considerable turmoil. To assume responsibility for our affairs at any time requires an extraordinary commitment to work on behalf of the Society and dedication to our scientific discipline. To take responsibility at that particular time also required a great deal of courage. This last point was emphasized by Mr. Arly Allen, President of Allen Press, who has written to commend our Secretary-Treasurer for the excellent job he has done. Allen Press handles our business affairs as well as our publishing activities, so Mr. Allen fully appreciates the difficult circumstances under which Dr. Schmidt took office. Our Society is now in excellent condition, financially and otherwise, and we are all deeply indebted to our Secretary-Treasurer for the large part he has played in bringing this about. The thanks and best wishes of the Society were expressed by acclamation.

The report was ordered placed on file.

B. Secretary-Treasurer (G. D. Schmidt)

Communications from the Secretary-Treasurer's office to the membership from July 1986 through June 1987 included a call for nominations for the Henry Baldwin Ward Medal, and a report from the Nominating Committee in the form of a ballot and biographical information on the candidates for 1988 Officers and Nominating Committee.

Communications with the Officers and Council Members-at-Large that required action included:

1. Three lists, totaling 68 applications for membership in the Society from July 1986 through December 1986, and four lists, totaling 55 applications from January 1987 through June 1987, making a combined total of 123 members. All were approved.
2. Approval of the 1987 Stoll-Stunkard Endowment Fund Speaker, Dr. Leroy Hood, California Institute of Technology.

TABLE I. *American Society of Parasitologists, Inc. statement of financial condition at 31 December 1984, 1985, 1986, and 31 May 1987.*

	1984	1985	1986	5 31 87
Current Assets:				
Category I	$121,836	$122,419	$159,400	$127,493
Category II	152,182	229,241	301,505	415,161
Category III	55,899	48,678	124,619	52,710
	$329,917	$400,338	$586,524	$595,364
Liabilities:				
Category I	$ 2,116	$ 5	$ 0	$ 0
Category II	451	0	0	583
Category III	12,000	0	0	0
	$ 14,567	$ 5	$ 0	$ 583
Fund Balances:				
Category I	$119,720	$122,414	$159,400	$127,493
Category II	151,731	229,241	302,505	414,578
Category III	43,899	48,678	124,619	52,710
	$315,350	$400,333	$586,524	$594,781
Net Worth	$329,917	$400,338	$586,524	$595,364

3. Approval of the 1987 Henry Baldwin Ward Medalist, Dr. Bruce M. Christensen, University of Wisconsin.
4. Approval of page charges for *The Journal of Parasitology* to be $45.00 for members, after five free pages, and $70.00 for nonmembers, who will be invited to join the Society. Exceptional cases may be waived, at the discretion of the Editors.

As of 1 July 1987, the Society had a total membership in good standing of 1,418. This includes 1,074 Active Members, 47 Active Nonsubscribing Members, 175 Student Members, 49 Retired Members, 42 Retired Nonsubscribing Members, 15 Emeritus Members, and 14 Honorary Members (including 1 Honorary/Active Member, and 1 Honorary/Retired Member). In May 1986 the total membership was 1,457. In addition to members, as of 1 July 1987 there were 1,509 Institutional Subscribers, down 47 from 1 July 1986.

Council approved 74 applications for membership in 1979, 53 in 1980, 116 in 1981, 96 in 1982, 128 in 1983, 93 in 1984, 116 in 1985, 113 in 1986, and 55 through June 1987.

Active Nonsubscribing Members must be a spouse of an Active Member. The name of the spouse will now be entered on the dues notice form.

The Society's contract with Allen Press, approved at the December 1986 Annual Meeting, was revised by Allen Press for 1987, with no price increase made for 1987. The contract for 1987 is now the same as for 1985 and 1986.

The financial records of the American Society of Parasitologists were prepared by Weatherwax & Roark, P.A., Certified Public Accountants, Lawrence, Kansas. The 1986 audit was conducted by Mize, Houser & Company, P.A. (previously named Barrand Eagan and Company), Lawrence, Kansas, at a cost of $1,375.00.

A statement of financial condition, summarizing current assets, liabilities, and fund balances at 31 December 1984, 1985, 1986, and 31 May 1987 is given in Table I. The Society's net worth at the end of May 1987 is $595,364, compared with $244,605 in December 1981.

A petty cash fund was continued by the Secretary-Treasurer's office. Its records have been examined and approved by the Auditing Committee.

The supply of certificates for the Ward Medalists and Emeritus and Honorary Members was about depleted. A printer was found who replenished them for a very minimal amount. The last Ward Medal on hand will be presented this year. A new supply has been ordered.

The late fee for tardy dues payment was instituted this year. Many paid; no complaints were received.

Profits from the 1986 Annual Meeting were $5,363, plus return of $1,000 seed money.

The Society traded advertising with the American Society of Zoologists and subscription forms with the American Microscopical Society.

The report was ordered placed on file.

C. Editors (D. F. Mettrick and S. S. Desser)

Following the December 1986 meeting of the Society, Allen Press agreed to copy edit our manuscripts starting in January 1987. We are pleased to report that our back-log has now been eliminated. This means that by October the *Journal* should start to appear in the appropriate month and that the turnaround time for good papers could be as short as 6 months. This change, however, means that authors must assume a greater responsibility for the accuracy of their papers. Do not just address the questions asked by the Editors and/or Allen Press. Check the entire galley proof carefully for errors.

The review articles have been well received. We would appreciate suggestions from the membership for additional timely articles, including the names of appropriate authors.

In order for Letters to the Editors and Critical Comments to be abstracted, please provide appropriate titles for your submissions.

You will have noticed that some advertisements have appeared in the *Journal* recently. These are submitted in copy-ready form and provide an additional source of income to the Society.

The new publication charges may result in a reduction in papers from foreign (nonmember) parasitologists. This reduction may be counterbalanced by the rapid turnaround time for manuscripts, which should make the *Journal* more attractive to authors.

We would remind authors that, before submitting a paper for *The Journal of Parasitology,* they should read the Instructions for Authors. In particular, there are more errors in Literature Cited than any other part of the manuscript. Secondly, all plates must be mounted on hardboard, indicating top, name of author, and manuscript number. Index descriptors are often missing (not required for Research Notes). Another problem concerns the title of the paper. Frequently the title does not clearly indicate what the paper is about; if the reader can not understand the title, they are unlikely to read your paper. Please make sure that the title of your paper does indeed indicate the nature of your article.

As our term as editors will end in 17 months, we would like to remind the membership that thought should be given to our replacements. We would suggest, from our experience, that having two co-editors, preferably from the same department, works out well. Over a 5-year period, a co-editor may become ill or be on sabbatical leave for part of the time. Holidays result in a mountain of manuscripts, reviews, revisions, etc. This work requires considerable time on a day-today basis, and is best shared between two otherwise busy individuals.

The report was ordered placed on file.

Accountant's Report

As previously reported, I have used the same procedures and tests that I consider necessary for monthly accounting and auditing of *The Journal of Parasitology* account.

- No salary or benefits of an editorial assistant have been encumbered for the month of June 1987.
- Other supply expenditures are recorded and processed on a daily basis.

In my opinion, the enclosed financial statement represents the straightforward financial position of the above account, the balance of which is Can $8,494.56 and is accurate and correct as of 31 May 1987. This report of operations for the year thus ended is in accordance with University of Toronto accounting principles.

Journal of Parasitology, Trust Account #3-436-120-50: Statement of income and expenditures for the period 1 June 1986 to 31 May 1987.

Income

Balance forwarded 1985/1986	Can $ 3,381.11
American Society of Parasitology	30,373.18
Total Income	$33,754.29

Expenditures

Salaries		
Editorial assistant	15,178.57	
Benefits	1,895.14	
Secretarial assistants	1,959.60	
	19,033.31	
Bi-monthly expenses (postage, xerox, telephone, computer time, etc.)	4,316.11	
Delivery	1,500.35	
Stationery	121.83	
Printing	288.13	
		25,259.73
Balance, 31 May 1987		Can $ 8,494.56

Peter Thinh, Accountant

The report was ordered placed on file.

D. Program Officer (R. W. Mead)

The 62nd Annual Meeting of the American Society of Parasitologists is scheduled to be held jointly with the Wildlife Disease Association, 2–5 August 1987. The meeting will begin with an opening session scheduled for 11:00 A.M. 3 August, and it will be presided over by William C. Campbell, President, American Society of Parasitologists. The Meeting of the Society consists of 121 presentations. Of these, 92 will be presented in 12 sessions; 21 will be displayed in a poster session; and five will be presented at the President's Symposium entitled "Strategies for Success in Helminth Life Cycles." The opening session will include an invited opening lecture that will be delivered by K. Darwin Murrell (Animal Parasitology Institute, Beltsville). The Stoll-Stunkard Endowment Fund Lecture is scheduled for Monday afternoon and will be presented by Leroy Hood (California Institute of Technology, Pasadena). The fourth R. B. McGhee Memorial Lecture will be presented by J. F. A. Sprent (University of Queensland, Brisbane, Australia), and it is scheduled for Tuesday afternoon. Dr. William C. Campbell will give the Presidential Address which is entitled "Heather and Ice: An Excursion in Historical Parasitology." This address will be presented Wednesday morning. The program will also include presentation of the Henry Baldwin Ward Medal and the Annual Business Meeting.

This is the second year that the Annual Program of the Society has included a Student Paper Competition. Three sessions are devoted to this competition which includes 22 presentations.

The Local Committee has arranged for an evening joint reception on Monday at 7:00 P.M., a reunion of Ward Medalists on Tuesday evening at 7:30 P.M., and morning and afternoon coffee breaks. The Society banquet is scheduled for 7:00 P.M. Wednesday evening.

Copy was submitted to Allen Press on 16 April. Proofs were received on 15 May, and corrected proofs were returned the same day. It is expected that the *Program* and *Abstracts* will be mailed to members by mid-June. Copies of abstracts were mailed to chairpersons on 12 May. Postcards indicating acceptance of abstracts were mailed as abstracts were received. Approximately one-

fourth of authors availed themselves of the opportunity to submit self-addressed stamped postcards on which they would be informed of the time their papers were to be presented. These cards were mailed on 12 May.

The report was ordered placed on file.

E. Archivist (M. H. Pritchard)

Additional records have been received and added to the Society's Archives. The catalogue of materials continues.

The report was ordered placed on file.

II. REPORTS OF STANDING COMMITTEES

A. Awards (J. A. Starling and W. M. Kemp, Co-chairmen; L. R. Ash, D. E. Sonenshine, and J. F. Williams)

The Committee considered five outstanding nominees for the 1987 Henry Baldwin Ward Medal. The recommendation of the Committee nominating Dr. Bruce M. Christensen as the recipient of the 1987 Henry Baldwin Ward Medal was forwarded to Council for approval.

The report was ordered placed on file.

B. Business Advisory (H. G. Sheffield, Chairman; D. D. Cox, J. S. Mackiewicz, P. H. Silverman, and G. D. Schmidt, ex officio)

The Society did well during 1986. At the end of the year our net worth was $480,207.79, which represents an increase over 1985 of $80,362.85 (20%). It is anticipated that our financial condition will be relatively stable during 1987 and no dues nor subscription rate increases are recommended.

The members of the Committee have been reviewing the monthly financial statements provided by the accounting firm and have not found any apparent discrepancies.

Subscriptions are down slightly, as anticipated by the Committee last year, but membership has been stable. Changes in the tax laws have not precipitated a loss of members.

The Committee has considered the issue of having a reduced rate membership with no journal subscription. Such membership is not recommended since it would provide relatively little benefit to the members and would not be advantageous to the Society.

The report was ordered placed on file.

C. Education (G. P. Noblet, Chairman; D. R. Brooks, P. A. D'Alessandro, J. C. Holmes, H. A. James, and G. A. Schad)

Members of the Education Committee who are attending the 1987 ASP meeting in Lincoln, Nebraska, are scheduled to meet during that time to discuss possible action to be taken in the future.

The report was ordered placed on file.

D. Honorary and Emeritus Members (J. Janovy, Jr., Chairman; R. Fayer, and A. J. MacInnis)

The 1987 report of the Committee on Honorary and Emeritus Members is in two parts: (1) a recommendation that Dr. O. N. Bauer be elected into Honorary Member status, and (2) draft guidelines for the selection of individuals who will be honored with a commemorative issue of the *Journal.*

1. The Committee on Honorary and Emeritus Members submits to ASP Council the name of Dr. Oleg Nikolaevic Bauer, Zoological Institute Academy of Sciences Leningrad, U.S.S.R., as its nominee for Honorary Membership. Dr. Bauer has been an active international collaborator with others whose interests lie in the parasites of fishes, and, through his efforts as author, coauthor, or editor, has made large segments of the Russian fish disease literature available through translation into English. The most familiar of Dr. Bauer's works include "The key to the parasites of fishes of the U.S.S.R.," "Diseases of pond fishes," and "Parasites of freshwater fish and the biological basis for their control." His major contribution to the American Society of Parasitologists has thus been made by use of his multilingual abilities to satisfy the need he recognized, namely that of providing access to a large body of Russian literature through international collaboration and translation.

2. Draft guidelines for the selection of individuals who will be honored with a commemorative issue of *The Journal of Parasitology*:

The ASP Committee on Honorary and Emeritus Members has the responsibility of recommending to ASP Council the names of individuals who will be honored by a commemorative issue of *The Journal of Parasitology.* The proposed guidelines and criteria for selection are as follows:

a. The nominee should be a member in good standing of the American Society of Parasitologists. Rationale: *The Journal of Parasitology* is a publication of ASP and the Society exerts control, through the Editor, over the various items of business and scientific policy that are associated with the *Journal.* A commemorative issue adds historical and social material to the scientific contents of the *Journal.* While the scientific contents must remain an open forum to which any individual may submit research results for review and possible publication, the social and historical contents are much more the property of the Society and should be used to acknowledge major contributions by a Society member. The word "should" allows the Committee to make exceptions to the membership criterion, but such exceptions would likely be very well documented and involve highly appropriate nominees.

b. The nominee must have made a lengthy and major contribution to the field of parasitology, although it is recognized that this contribution can take several forms. The supporting materials submitted with the nomination should make it clear that the nominee has had an identifiable impact on the general field of endeavor known as "parasitology" in the broad sense. Rationale: A commemorative issue is a great honor; there is no need to honor an individual who has not made a significant contribution to the field of parasitology. However, it should be recognized that over the course of a long professional career, there are many ways a person can influence the manner in which a field of endeavor is pursued, for example through teaching, research, the administration of research, service to ASP, etc. A general guideline for nominees is that

one should be able to say about them: If — had not done —, then the field of parasitology would be much poorer than it is now.

c. Commemorative issues of *The Journal of Parasitology* should occur with a frequency of one every three to five volumes. Rationale: The extent to which a commemorative issue is an honor is determined by the supply of such issues. This guideline is intended to hold until the Committee on Honorary and Emeritus Members obtains a sense of how many nominees there are likely to be for commemorative issues.

d. A commemorative issue will contain a title on the cover, a portrait of the honoree, and a summary of his or her contributions, typically written using excerpts from supporting materials submitted with the nomination. It is appropriate to include in the summary a selected list of publications. Rationale: If the honor is restricted to the cover, a portrait, and a summary, then the editorial problems associated with the honor are minimized. If the honor is accompanied by special papers, then the Committee is charged with the solicitation of these papers (an inappropriate activity for the Committee), which would then have to be reviewed, revised, and perhaps rejected. It is in the best interests of the *Journal* to separate its scientific functions from its historical and social ones—thus the restriction of a commemorative issue to a title page, a portrait, and a summary.

e. The summary to be printed in the *Journal* may be prepared by the Committee on Honorary and Emeritus Members, or by a person selected by the Committee after consultation with the Editor.

f. Nominations for honor by commemorative issue must be well documented and must include biographical material, a list of the most significant publications or other specific contributions, and a short statement of the reasons why this particular nomination is especially appropriate.

The report was ordered placed on file.

E. In Memoriam (J. L. Bennett, Chairman; R. J. Boisvenue and C. E. Tanner)

Mr. President, we report with regret the death of the following members:

DR. CECIL A. HOARE, date unknown;
DR. WILLIAM B. LEFLORE, 6 December 1986;
DR. CORNELIUS B. PHILIP, on 8 January 1987;
DR. JOSEPH E. SEMRAD, date unknown.

We request a moment of standing silence in memory of our departed colleagues.

The report was ordered placed on file.

F. Membership (D. J. Forrester, Chairman; W. C. Campbell, A. Flisser, and J. W. McCall)

No activity.

The report was ordered placed on file.

G. Nomenclature and Terminology (M. H. Pritchard, Chair; B. M. Honigberg, J. R. Lichtenfels, J. S. Mackiewicz, and B. J. Myers)

No questions have been referred to the Nomenclature and Terminology Committee this year. The proposed National Biological Survey is still a matter of discussion, and the illustrated glossaries are progressing slowly.

The report was ordered placed on file.

H. Nominating (C. W. Kim, Chairman; D. G. Dusanic, D. M. Dwyer, R. B. Grieve, T. R. Klei, and M. H. Dresden, Alternate)

Nominating Committee members were contacted either personally at the 61st Annual Meeting of the American Society of Parasitologists at Denver (7–11 December 1986) or by letter on 15 December 1986, congratulating them as members of the Nominating Committee for 1987.

On 30 December 1986, committee members were requested by letter to submit nominees for the following positions: President-Elect, Vice-President, Secretary-Treasurer, Council Members-at-Large, and Nominating Committee.

On 28 January 1987, committee members were requested by letter to prioritize (rank) each nominee in each category from a long list of nominees submitted by committee members in December. This was done in the interest of time since the December meeting at Denver was an unusually late meeting, which did not allow sufficient time for another round of ballots.

The nominees were selected according to their prioritization and contacted by telephone and in writing. Upon their acceptances, their names were listed on the final slate.

On 24 February 1987, members of the Nominating Committee and the Secretary-Treasurer were informed in writing of the final slate of nominees.

On 6 March 1987, the following list of nominees with accompanying camera-ready biographical sketches were transmitted to the Secretary-Treasurer for use in the official ballot:

President-Elect 1988
- Catharine Crandall
- W. Michael Kemp

Vice-President 1988
- John H. Cross
- K. Darwin Murrell

Secretary-Treasurer 1988–1990
- Lillian F. Mayberry
- Clarence A. Speer

Council Members-at-Large 1988–1991
- Lawrence Ash
- Michael D. Ruff
- John R. Seed
- Jeffrey F. Williams

Nominating Committee 1988
- Roy C. Anderson
- Dwight Bowman
- Richard B. Crandall
- Philip A. D'Alesandro
- Barbara Doughty
- Stephen G. Kayes
- James B. Lok
- John W. McCall
- Charles E. Tanner

The report was ordered placed on file.

I. Public Responsibilities (J. H. Oliver, Jr., Chairman;

P F Basch, E M Cornford, R B Crandall, and K G Powers)

The Public Responsibilities Committee has nothing to report at this time.

The report was ordered placed on file.

J. Special Awards (B. B. Nickol, Chairman; J. B. Jensen and D. C. Kritsky)

The paper entitled "Phylogenetic and developmental studies of *Glypthelmins shastai* and *Haplometrana intestinalis*, two digenean gut parasites of anurans in western North America," presented by R. T. O'Grady at the 1986 ASP meeting, was selected to be the Society's nomination for the 1987 Best Student Paper in Systematics Award sponsored by the United States Federation of Culture Collections. The late 1986 meeting date of ASP made it necessary to have this nomination considered for the 1987 award to be announced early in 1988. A paper presented at the 1987 meeting will be nominated for the 1988 award.

The report was ordered placed on file.

K. Stoll-Stunkard Endowment Fund (S. G. Kayes, Chair; R. W. Mead, P. T. LoVerde, and R. M. Overstreet)

The committee, under the chairmanship of S. G. Kayes selected Dr. Leroy E. Hood, California Institute of Technology to present the Stoll-Stunkard Endowment Fund Lecture for the 1987 Annual Meeting. The committee is now in the process of selecting a speaker to recommend to the Council for the 1988 meeting.

The committee was charged by Council to assume responsibility for choosing the R. Barclay McGhee Memorial Lecturer for the 1987 Annual Meeting. In consultation with the Parasitology Faculty of the University of Georgia, Dr. J. F. A. Sprent, Emeritus Professor of Parasitology, University of Queensland, Brisbane, Australia, was chosen to present the R. B. McGhee Memorial Lecture at the 1987 Annual Meeting. The committee is now in the process of selecting a speaker to recommend to the Council for the 1988 meeting.

L. Tellers (T. Nolan, Chairman; L. Slayton and R. Wendt)

The Secretary-Treasurer forwarded 634 ballots to Thomas Nolan, Chairman. A detailed tally was submitted to the Secretary-Treasurer. The results of the ballot for Officers, Council Members-at-Large, and Nominating Committee are as follows:

President-Elect (1988)
- Catharine A. Crandall
- W. Michael Kemp*

Vice-President (1988)
- John H. Cross
- K. Darwin Murrell*

Secretary-Treasurer (1988–1990)
- Lillian F. Mayberry*
- Clarence A. Speer

Council Members-at-Large (1988–1991)
- Lawrence R. Ash*
- Michael D. Ruff
- John R. Seed
- J. F. Williams*

Nominating Committee (1988)
- Roy C. Anderson* (Chairman)
- Dwight D. Bowman
- Richard B. Crandall (Alternate)
- Phillip A. D'Alesandro*
- Barbara L. Doughty*
- Stephen G. Kayes
- James B. Lok
- John W. McCall*
- Charles E. Tanner*

The report was ordered placed on file.

M. Translations (A. Raikhel, Chairman; F. G. Hochberg, G. L. Hoffman, M. N. Lunde, and L. Margolis)

Forty-two titles of new translations have been submitted by Committee Members. A total of 122 titles have been accumulated since the last list of translations was published in 1983. A new list of translations for the past 5 years is being prepared, with completion expected by fall 1987.

The report was ordered placed on file.

III. REPORTS OF AD HOC COMMITTEES

A. Clinical Laboratory Standards (L. S. Garcia, Chairman; G. R. Healy)

During this past year we have been working closely with NCCLS to begin the process of drafting the NCCLS approved project: Collection, Processing, and Examination Procedures for the Recovery and Identification of Parasites from Fecal Specimens. Based on the fact that the committee will not actually have to meet as a group, we have received permission from NCCLS to begin the project without an official budget review process. Communication was sent to individuals who had expressed an interest in the project and we requested they respond by 1 July 1987.

We would like to encourage any interested member to contact Lynne Garcia and to send a copy of your resumé to her for submission to NCCLS. The size of the committee does not have to be limited and members can participate in the writing and/or review process. However, NCCLS requires a resumé on file before you can be considered a member of the committee. Resumés can be submitted until 31 December 1987.

Send to: Lynne S. Garcia, Clinical Microbiology, A2-250, UCLA Medical Center, Los Angeles, California 90024.

We would also like to encourage the members to contact us if they have any suggestions for other possible projects in the clinical area.

The report was ordered placed on file.

B. Local Committee 1987 Meeting (J. Janovy, Jr. and B. B. Nickol, Co-Chairmen)

No report was received.

C. Local Committee 1988 Meeting (G. W. Esch and R. E. Kuhn, Co-Chairmen)

The 63rd annual meeting of the ASP will be held at Wake Forest University in Winston-Salem, North Carolina, 1–4 August 1988. The local committee has been organized and has already met once. A second meeting is scheduled to coincide with the site visit of the Program Officer and the Secretary-Treasurer next fall. Ten-

* Elected.

tative plans include an evening banquet, a reception and barbeque dinner on another evening, and an all-day symposium entitled, "Patterns and Processes in Parasite Communities." We have also made arrangements for Piedmont Airlines to serve as our official air carrier; in return we will receive special discounts and other promotional services. Attendees will be housed in an air-conditioned residence hall on campus and within easy walking distance of all paper sessions and dining facilities.

The report was ordered placed on file.

D. Local Committee 1989 Meeting (D. R. Brooks)

No report was received.

E. Newsletter (L. K. Eveland, Editor)

The 1987 Newsletter staff: Editor, Vern Eveland: Associate Editors, Gilbert A. Castro (President-Elect), B. Fried, B. J. Myers, G. D. Schmidt (Secretary-Treasurer), and J. E. Ubelaker; Affiliated Society Correspondents, SCSP—E. G. Platzer, RMCOP—R. A. Heckman, SWAP—L. F. Mayberry, SSP—None, NCP—M. Norberg, NJSP—M. Doscher, AMCOP—D. M. Miller, HELMSOC—M. D. Ruff.

When report was submitted, Issue Numbers 1 and 2 of Volume 9 were published, and Issue Number 3 of Volume 9 was in preparation. Number 1 contained affiliated society news, ASP representatives reports, announcements of awards, new editorship for *Experimental Parasitology,* immunoparasitology symposium, scientific exchange, AIBS-ASPP congressional fellow, obituaries, members and friends, TEKMED, needs and available items, publications, positions available, and other miscellaneous. Number 2 contained a message from the president, announcements of affiliated society news, obituaries, members and friends, meetings, ASP officers, council members, committees, and representatives, Stoll-Stunkard endowment fund contributors, membership directory fund contributors, and other miscellaneous. Goals of the Newsletter are to publish items and editorials which: (1) are in the interest of members; (2) keep members informed about affiliated and other societies of parasitology; and (3) promote parasitology teaching, research, and clinical service.

The report was ordered placed on file.

F. Priorities in Parasitology (J. R. Lichtenfels, Chairman; R. T. Damian, J. K. Frenkel, and P. M. Schantz)

No report is available. See New Business: I. Priorities in Parasitology Committee.

G. Special Student Awards (B. Fried, Chairman; C. M. Bartlett, R. T. Damian, D. W. Duszynski, M. Grogl, R. Herman, A. D. Johnson, and A. K. Prestwood)

A total of 23 papers were submitted for the student paper competition at the 1987 ASP meeting. The papers were arranged in three student paper competition sessions consisting of 10, 9, and 4 papers. The presiding officers (who are also members of this committee) of each session will serve as primary judges of that session, along with other committee members as available. At the completion of the third session, members of the Committee will meet to determine the two student awardees. The Committee will use the method of scoring and judging devised by last year's Chairman, Dr. G. A. Castro. Dr. Castro's report (Journal of Parasitology, 1987, **73**: 26) has been very helpful to the Committee.

The report was ordered placed on file.

IV. OLD BUSINESS

A. Honoree Selection for Commemorative Issues of the Journal

The Honorary and Emeritus Members Committee's proposed guidelines and criteria for selection of individuals who will be honored with a commemorative issue of the *Journal* (see IID) was accepted by Council.

B. Joint Meeting with AIBS

Council voted not to meet with AIBS in 1992.

C. *Careers in Parasitology* Booklet Revision

Councilman Frenkel reported on the status of the revision of the *Careers in Parasitology* booklet. Progress has been made, and Dr. Frenkel requested more input from the membership.

D. Annual Meeting Theme Slogans

Theme slogans for future meetings (proposed by Harry Hoogstraal in 1985) was discussed. It was decided not to pursue the idea any further at this time.

V. NEW BUSINESS

A. Applications for Membership

It was moved and seconded that the applications of 13 new members (#5387–5399), duly nominated and seconded, be approved by Council. The motion carried.

B. Honorary Member Nomination

The Honorary and Emeritus Members Committee nominated Dr. O. N. Bauer to the status of Honorary Member. Council unanimously voted to accept the nomination.

C. Program Officer 1988–1990 Nomination

At the recommendation of Secretary-Treasurer-Elect Lillian F. Mayberry, President Campbell nominated Donald W. Duszynski as Program Officer, to replace Robert W. Mead, outgoing Program Officer. Council approved the nomination.

D. Editor Search Committee

President Campbell appointed an Editor Search Committee to find a replacement for the current Editors, whose terms of office expire 31 December 1988. Those appointed were: R. T. Damian (Chairman), R. J. Lichtenfels, P. T. LoVerde, A. J. MacInnis, and G. D. Schmidt.

E. Certificates for Speakers

At the suggestion of the Stoll-Stunkard Endowment Fund Committee (S. G. Kayes, Chairman), Council voted to provide certificates to future and past Stoll-Stunkard Endowment Fund speakers and R. Barclay McGhee lecturers. Secretary-Treasurer-Elect Mayberry will prepare and distribute them.

F. Reprint and Page Charges

Difficulties in collection of reprint and page charges were discussed by Council. Secretary-Treasurer Schmidt was directed to advise Allen Press that the Society will no longer be involved in the selling of reprints to authors.

G. Computer for Secretary-Treasurer's Office

Council voted to provide Secretary-Treasurer-Elect Mayberry with a computer for Society business.

H. NCCLS Affiliation

Continued affiliation with the National Committee for Clinical Laboratory Standards was discussed by Council. After an initial motion to discontinue affiliation, the motion was tabled, pending further input from the Society's representative, Lynne S. Garcia.

I. Priorities in Parasitology Committee

Upon the recommendation of J. R. Lichtenfels, Chairman, it was voted to retain the Priorities in Parasitology Committee. The Committee was directed to shift its emphasis to priorities of the Society, rather than priorities of parasitology as a whole. The name will be changed to "Priorities of the American Society of Parasitologists" Committee.

J. Special Student Awards Committee

It was voted to change the Special Student Awards Committee from an ad hoc committee to a standing committee. Secretary-Treasurer Schmidt and Secretary-Treasurer-Elect Mayberry were directed to proceed with the changes, as mandated by the Bylaws of the Society.

VI. ADJOURNMENT

President Campbell declared the Seventy-seventh Council Meeting adjourned at 2:00 P.M.

Respectfully submitted,
Gerald D. Schmidt
Secretary-Treasurer

STRATEGIES FOR SUCCESS IN HELMINTH LIFE CYCLES

President's Symposium

62nd Annual Meeting

American Society of Parasitologists

Lincoln, Nebraska, August 1987

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J. Parasit., 74(1), 1988, pp. 28–29

INTRODUCTION

J. Ralph Lichtenfels
Animal Parasitology Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

It has been my pleasure as ASP Vice President for 1987 to work with President Bill Campbell to organize and to introduce the President's Symposium.

In the preface to the third edition of his textbook, "Animal Parasites, Their Life Cycles and Ecology," O. W. Olsen listed 5 basic principles of parasitology essential to an understanding of the subject: (1) a basic classification for understanding relationships; (2) the general morphology of adults and larvae, for recognition; (3) the patterns of life cycles, for comprehension of how parasites live; (4) the ecological requirements of all stages of the parasites; and (5) the means of transmission of all stages.

I think we can agree that a knowledge of life cycles and transmission patterns are at the heart of parasitology. Indeed, I suspect that the marvelously ingenious strategies evolved by parasites for ensuring their survival probably had a role in attracting many of us to the field of parasitology.

The success of this symposium, as judged by the enthusiasm of the audience and the willingness of the editors to publish it, is due in large part to the superior contributions of the speakers. However, a contributing factor may be that our society includes many members who have not had classical training in parasitology or have been away from research in this sector of the parasitology spectrum for some time. Life cycles and transmission patterns provide a framework or practical reference point for all work in parasitology. An understanding of these central concepts in our field is necessary for all of us to communicate to peers and laymen alike the importance of our work.

The 3 contributions were not intended to be exhaustive reviews and authors whose work is not cited should not be offended. Each speaker

was limited to 30 min so only an overview with selected examples was possible.

The word strategies in the symposium title may give the appearance of anthropomorphic design on the part of the parasites. Readers, of course, must realize that successful strategies in life cycles are the result of time and selection pressures.

Each speaker approached his subject differently from the others, although all 3 provided new insights into the life cycles themselves, how they evolved, and what they were selected for. Professor Roy C. Anderson discussed the different strategies used by nematodes and then provided examples of different strategies in each of the superfamilies parasitic in vertebrates. He provided new insight into precocity (growth and/or development beyond the expected) and gave numerous examples of this phenomenon in nematode life cycles. He agrees with others who believe that intermediate hosts were added after the colonization of vertebrates by nematodes.

In contrast to the nematodes, the trematodes, as discussed by Dr. Wesley L. Shoop, evolved from a 1-host pattern in a mollusc to produce sequential generations in the mollusc and then added final, intermediate, or paratenic hosts to achieve complicated 3- and 4-host cycles. Dr. Shoop discusses the highly evolved transmammary transmission of *Alaria marcianae* from this evolutionary perspective.

Professor John S. Mackiewicz discussed the evolution of cestode life cycles and agreed with others who believe a 2-host cycle to be strongly selected for in nature over a 1-host cycle because of enhanced transmission through intermediate host dispersal. He concludes that transmission is usually by passive stages that become incorporated into the food chain of the host. Paratenesis as in nematodes and trematodes is important also in cestodes. Unlike trematodes, asexual proliferation of immature stages is uncommon in cestodes, but the evolution of methods of increasing egg production in the final host are common.

My final tasks are to congratulate the 3 speakers for acting as superb guides to the heart of our discipline; to thank president William C. Campbell for the wisdom to select this subject; and, to thank Editors David F. Mettrick and Sherwin S. Desser for recognizing the value of bringing these papers to the readers of our journal.

J. Parasit., 74(1), 1988, pp. 30–45

NEMATODE TRANSMISSION PATTERNS*

R. C. Anderson
Department of Zoology, College of Biological Science, University of Guelph,
Guelph, Ontario, Canada N1G 2W1

ABSTRACT: The transmission of nematode parasites of vertebrates is reviewed with special reference to the phenomena of monoxeny, heteroxeny, paratenesis, and precocity. Monoxeny is divided into 2 types. Primary monoxeny assumes that there was never an intermediate host in the transmission. Secondary monoxeny assumes the loss of an intermediate host during the course of evolution and its replacement by a tissue phase in the final host. Heteroxeny, or the use of intermediate hosts, is a common feature of many nematode groups. The Spirurida utilize arthropods, the Metastrongyloidea molluscs, and Ascaridida arthropods and vertebrates. Paratenesis, or the use of transport hosts, is a common feature of the transmission of nematode parasites of carnivores. It is postulated that in some instances paratenic hosts have become intermediate hosts and replaced the original intermediate host. Precocity in the development of nematodes in intermediate hosts (including what may have been paratenic hosts) is defined as growth and/or development beyond the expected. Its occurrence among the nematode parasites of vertebrates is reviewed. It is regarded as a transmission strategy which accelerates gamete production in the final host. Precocity could also provide the mechanism for the transfer of a parasite from a predator final host to a prey final host.

In the past 25 yr a great deal of information has accumulated on the transmission of parasitic nematodes, much of it widely scattered in the literature and not fully appreciated by parasitologists generally. The subject of transmission is important, not only because of its relevance to human and animal medicine, but also because an understanding of transmission can help explain how nematodes became parasitic in the first place and how they have managed to radiate in some host groups (and not in others) during the course of evolution.

There are about 16,000 described species of nematodes (Poinar, 1983) of which about 40% are animal parasites. In reviewing briefly the transmission of the parasitic forms it will only be possible to give a few highlights from an extensive literature which cannot be cited fully. About 8% of the known parasitic nematodes occur in invertebrates and these (e.g., the Tylenchida) sometimes have transmission patterns unparalleled among the parasites of vertebrates (Poinar, 1983). Except for a brief mention of the mermithoids, this review will be confined to the parasites of vertebrates.

GENERAL SYSTEMATIC ARRANGEMENT OF THE NEMATODES

For the convenience of the general reader the classification used herein follows the CIH keys (Anderson et al., 1974–1983) although there are recent proposals to alter the names and organization of some higher taxa (e.g., Adamson, 1987). In the CIH system the Adenophorea (Fig. 1) consists of 2 major groups, the marine Chromadorida and the Enoplida. The latter is divided into the freshwater and marine Enoplina and a predominantly soil group, the Dorylaimina. The Dorylaimina, which includes the soil-dwelling dorylaimoids, gave rise to 4 small parasitic lines including the mermithoids, mainly of insects, and the trichinelloids, dioctophymatoids, and muspiceoids of vertebrates.

The Secernentea (Fig. 1) is widely represented in soil as about half of all soil nematodes belong to the Rhabditina. The Secernentea also contains many parasites of vertebrates and invertebrates derived from soil-dwelling rhabditids. It is believed the rhabditine line of soil nematodes also gave rise to the tylenchids, which are mainly plant parasites (Siddiqi, 1985). Although, the tylenchids successfully colonized terrestrial invertebrates, mainly the insects, they were never successful in invading vertebrates (1 species, *Myoryctes wiesmanni* Ebert, 1863, has been reported from a frog in Europe).

BROAD HOST DISTRIBUTION OF NEMATODE PARASITES

The most striking characteristic of the nematode parasites is the overwhelming dominance of the secernenteans over the adenophoreans and it is generally agreed that the rhabditid-line gave rise to about 92% of the parasites found in man and animals.

* President's Symposium on Strategies for Success in Helminth Life Cycles, American Society of Parasitologists Annual Meeting, 2–5 August 1987, University of Nebraska at Lincoln, Nebraska.

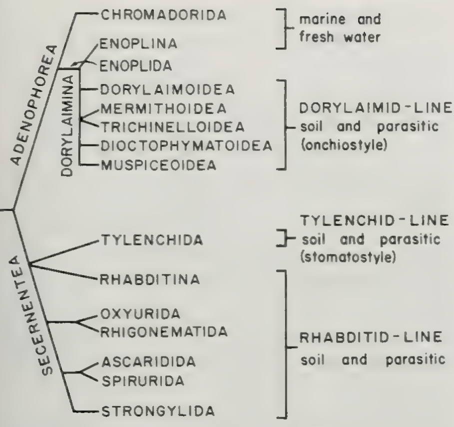

FIGURE 1. Arrangement of the higher taxa of the Nematoda based on the CIH keys to the nematode parasites of vertebrates edited by Anderson et al. (1974–1983).

Since both the parasitic secernenteans and adenophoreans can be traced back to soil ancestors, it has been hypothesized that parasitism by nematodes could not have occurred until animals invaded land, first the invertebrates, and much later the tetrapods (Anderson, 1984). This explains why nematode parasites are practically nonexistent in such important marine and freshwater groups as the molluscs, polychaetes, and crustaceans, and it explains the richness of the nematode fauna in essentially terrestrial groups like the earthworms and insects, and the terrestrial molluscs.

Where then did fishes acquire their nematodes? Anderson (1984) hypothesized that the fish nematode fauna was derived from nematodes that had already become parasites of early land vertebrates and that had developed certain transmission strategies which allowed some of them to transfer to, and radiate in, the fishes. The most important strategies were undoubtedly heteroxeny (the acquisition of intermediate hosts) and paratenesis (the use of transport hosts). These are the mechanisms found in the transmission of practically all the nematode parasites of fishes.

The nematode fauna of marine mammals is basically similar to that of the terrestrial mammals and one assumes that heteroxeny and paratenesis developed under terrestrial conditions allowed a limited parasitic fauna of nematodes to persist in seals and whales. Undoubtedly many species were lost during the transition to the aquatic milieu.

Nematode parasites are extremely rare in marine and freshwater invertebrates although the mermithoids, which are believed to have originated from the predaceous terrestrial dorylaimids and which occur in terrestrial insects, were able to transfer to aquatic insects, echinoderms, crustaceans, and other marine invertebrates. They achieved this transfer without the benefit of heteroxeny and paratenesis. The mermithoids are unusual in that larval stages are parasitic and the adult stage free-living and highly active (Poinar, 1983). The females lay their eggs near potential hosts. Presumably, mermithoids first developed parasitism in terrestrial hosts and then invaded freshwater and later the marine ecosystem by this simple system of transmission, which is so unlike that of other nematodes. The mermithoids have even been able to adapt to wasps and spiders, which do not breed in water or soil, by the use of insect paratenic hosts that are prey of wasps and spiders.

DEVELOPMENT OF NEMATODES

Maupas (1899, 1900) noted that rhabditid nematodes in culture passed through 5 stages separated by 4 molts and that when cultures became exhausted all stages died except those at the beginning of the third stage. These latter retained the cuticle of the second stage. The larvae, now known as dauer larvae, initiated new populations when placed in fresh cultures. Maupas noted that dauer larvae can survive for long periods of food scarcity and dry conditions.

Maupas suspected all nematodes went through 4 molts and subsequent work has shown this to be true even of the parasitic forms. Special significance can also be attached to the dauer larva of the rhabditids which is not only adapted to survive adverse conditions, but is also the dispersal stage, often making use of phoresy on the bodies of insects and even small mammals to find new food sources in which to develop. Osche (1963) has explored the role of the dauer larva in dispersal.

In the secernenteans it is generally the early third stage which is the infective or invasive stage. Chabaud (1954, 1955) related this rule to the origin of secernenteans from free-living rhabditids similar to those studied by Maupas. As will be seen, a number of secernenteans of vertebrates break the rule of the third infective stage but these exceptions are best interpreted as specializations from the primitive condition.

As indicated earlier, adenophoreans are de-

rived from soil-dwelling predaceous dorylaimids which lack dauer larvae. Thus, in the trichinelloids, dioctophymatoids, and mermithoids, the rule of the infective third stage does not pertain. These forms infect the host in the first as well as the third or fourth depending on the species.

PRINCIPLES OF TRANSMISSION

It was first suggested by Fülleborn (1920) that cutaneous penetration is the primitive mode of infecting the final host and Adamson (1986) has postulated that only those groups that arose from ancestors infecting the host in this way developed tissue parasitism and the use of intermediate hosts. Skin penetration almost always necessitates a tissue migration to the gut or some other definite site in the body.

Monoxeny

A number of major groups of nematodes are monoxenous in that they infect the final host directly. However, monoxeny can be of 2 types. In the first, which we shall term *primary monoxeny,* there is no reason to believe there was ever during the course of evolution an intermediate host in transmission. Monoxeny displayed by the trichostrongyloids, ancylostomatoids, and oxyuroids is of this type.

Secondary monoxeny assumes the loss of an intermediate host during the course of evolution. Fülleborn (1927) interpreted the tissue phase in *Ascaris lumbricoides* as an event that once took place in an intermediate host which has been abandoned. In other words the final host serves also as an intermediate host. This concept is useful for interpreting the transmission of ascaridoids. Secondary monoxeny is important in ascaridoids because it necessitates visceral larva migrans which may then lead to transplacental transmission as found, for example, in the genus *Toxocara.*

Heteroxeny

Many modern nematode superfamilies are heteroxenous. Intermediate hosts in the Spirurida are generally arthropods. Members of the Metastrongyloidea use oligochaetes and molluscs. The Ascaridoidea, on the other hand, utilize both vertebrates and invertebrates as intermediate hosts although Anderson (1984) regards the vertebrate as the primitive host.

It is generally agreed that nematodes were not originally parasites of what are now intermediate hosts. The most compelling argument in favor of this conclusion is that arthropods, for example, lack a nematode fauna independent of, but related to, major heteroxenous nematodes like the Spirurida. This is, incidently, in contrast to the Oxyuroidea, which are richly developed in both terrestrial arthropods and vertebrates. We conclude, therefore, that intermediate hosts were adopted only after ancestors of most heteroxenous nematodes had already established themselves as monoxenous parasites of vertebrates. The advantages of utilizing intermediate hosts have been enumerated by various authors including Chabaud (1954, 1955) and Inglis (1965).

Paratenesis

Many nematodes also use in transmission a transport or paratenic host (Baer, 1951) in which development usually does not occur and which functions to move larval stages through the food chain leading to the final host. Beaver (1969) coined the term paratenesis for this process.

Paratenesis occurs in 3 ways in the nematodes. Firstly, it may move larval stages from the external environment to the intermediate host in which development to the stage infective to the final host occurs. Secondly, it may move larvae between the intermediate and final hosts, thus carrying infective larvae to the final host. Thirdly, it may move larvae from one paratenic host to another.

To interpret the transmission of some groups of nematodes it becomes necessary to postulate that the paratenic host has become an intermediate host itself and often, during the course of evolution, replaced the original intermediate host. This hypothesis will be used later in an attempt to explain the unusually varied and complex transmission of ascaridoids.

Precocity

A number of nematode parasites develop beyond what one would expect in intermediate hosts. For example, secernenteans primitively develop to the early third stage and are then infective to the final host. We are aware of several examples where development proceeds beyond the early third stage and even up to the adult stage. This phenomenon will be called *precocity* and is defined as *growth and/or development beyond the expected.* Precocity is not uncommon among certain nematodes of fishes, mainly the Ascaridoidea and Spirurida, and it may often be associated with final host behavior, which re-

stricts transmission to reduced limits of time and space. Precocious development in intermediate hosts is likely an important strategy which accelerates gamete production in the final host.

Precocity (which, it should be noted, may also occur in what was primitively a paratenic host) can be manifested in 2 ways: (1) The parasites may develop beyond the stage to be expected, as for example, the early third stage in secernenteans, and (2) the parasite may remain in the expected stage, for example the early third stage in the secernenteans, but exhibit unusual growth without molting beyond the expected stage.

The evolution of *extreme precocity* (i.e., development to the adult stage in the intermediate host) as a transmission strategy in a group like the Ascaridoidea, which often use vertebrate intermediate hosts, might be a mechanism whereby a parasite could come to inhabit as an adult what was formerly the intermediate host, thus eliminating the need for the original final host which could then be dropped. This type of capture (for definition of capture see Chabaud, 1981) must be nonexistent or extremely rare in the Spirurida which use only arthropod intermediate hosts in transmission. Otherwise one would expect to find representatives of the Spirurida living independently in arthropods. However, if a vertebrate paratenic host becomes involved in the transmission of spirurids and precocity occurs in this host (i.e., it became an intermediate host) it might lead to capture by the mechanism described above. This might, for example, explain the broad host distribution of the Physalopteroidea.

Precocious development is well known among some digeneans where it can lead eventually to progenesis, or the production of gametes in larval stages (Schell, 1985).

THE ADENOPHOREANS

Dioctophymatoidea

The dioctophymatoids are heteroxenous and utilize aquatic oligochaetes as intermediate hosts. Third-stage larvae of *Dioctophyma renale* are directly infective to mink and the prepatent period is about 3 mo (Mace and Anderson, 1975). However, the ingestion of frog and fish paratenic hosts with third-stage larvae is the usual method of infection.

A species in a closely related genus, *Eustrongylides tubifex,* elicits large tumors in the stomach of fish-eating birds. It develops to the third stage in oligochaetes but the parasite must develop to a fourth stage in a fish paratenic host before it becomes infective to the final host. This system has been studied experimentally and in the field, and it seems evident that precocial development in the fish contributes to the exceedingly rapid maturity of the parasite in the bird (Measures, 1987). Indeed in 12 days large tumors have developed and the worms are shedding eggs into the proventriculus. This accelerated development is in contrast to *Dioctophyma renale* and is apparently an adaptation to a migratory avian host which spends only a few weeks in fall and/or spring on a specific body of water where transmission must occur in a short period of time.

Trichinelloidea

Species of *Capillaria* (*sensu lato*) and related genera are widespread in a great many vertebrates including fishes (Moravec, 1982; Moravec et al., 1987). Typically, the species produce unsegmented eggs that leave the host and embryonate in the external environment into first-stage larvae. In many species these eggs are infective to the final host. *Capillaria hepatica* is typical except that the eggs which are encapsulated in the liver must be released by carnivorism, often by cannibalism (Freeman and Wright, 1960). Other species use oligochaete paratenic hosts which may or may not be physiologically required. A molt is said to occur in the oligochaete in some species.

Capillaria philippinensis, in most respects a typical *Capillaria,* shows the potential of the group. Fish that ingest larvated eggs are apparently obligatory paratenic hosts. Larvae from fish readily infect primates, gerbils, and birds and grow to the adult stage in the intestine. Female worms produce thin-shelled larvated eggs that are auto-infective and typical thick-shelled eggs that are passed in the feces of the host and infect fish (Cross and Basaca-Sevilla, 1983).

The example of *C. philippinensis* may help to explain how *Trichinella spiralis* might have originated from a capillariid-like ancestor which evolved the ability to deposit larvae into the host's tissues.

Muspiceoidea

The biology of the muspiceoids is not fully understood. They are tiny, highly specialized adenophoreans inhabiting tissues of marsupials, birds, mice, and bats. Brumpt (1930) studied *Muspicea borelli* in mice many years ago presumably because it was thought the infections

were related to cancers. Transmission is suspected to be by cannibalism, cutaneous penetration, or during grooming or lactation (Bain and Chabaud, 1979; Spratt and Spears, 1982).

THE SECERNENTEANS

Rhabditoidea

The most common rhabditoids in vertebrates are *Rhabdias* of amphibians and reptiles, *Strongyloides* in a great variety of terrestrial vertebrates, and *Parastrongyloides* of rodents, insectivores, and marsupials. The parasitic stages are so highly modified that their designation as rhabditoids would be impossible. However, they have free-living generations which by their morphology clearly reveal their taxonomic affinities as the most primitive of the secernentean parasites of vertebrates.

In *Rhabdias* the parasitic form is a hermaphrodite that produces larvae that develop into the free-living *Rhabditis*-like generation in the waste of the host. Third-stage larvae of the free-living generation penetrate the skin of the host (Baker, 1979).

In *Strongyloides* the parasitic form is a parthenogenetic female. The free-living generation includes males and females that eventually produce infective larvae that invade the skin of the host and undertake a lung migration (Nigon and Roman, 1952).

In *Parastrongyloides* the parasitic forms consist of both males and females and the free-living generation resembles that of *Strongyloides* (see Mackerras, 1959).

The bursate nematodes (Strongylida)

The bursate nematodes constitute a very large group of parasites found in all vertebrates except the fishes. As is well known, the Diaphanocephaloidea, Trichostrongyloidea, Ancylostomatoidea, and Strongyloidea are monoxenous whereas the Metastrongyloidea are basically heteroxenous.

The primitive pattern of transmission is found in the monoxenous groups (see Durette-Desset, 1985, for a review of the Trichostrongyloidea). Eggs develop in the external environment and the larvae feed, grow, and undergo 2 molts. The second cuticle is retained on the early third stage, which is homologous to the dauer larva of free-living rhabditids. Typically larvae invade the skin of the host and undergo a lung migration on their way to the definite site in the gut.

In more evolved forms, the free-living stage is shortened by the occurrence of 1 or 2 molts in the egg. Also, oral transmission appears. In several species both oral and skin penetration may occur and this is followed by the typical lung migration. Finally, in the most evolved system skin penetration is replaced entirely by oral infection and the lung migration is eliminated.

Ollulanus tricuspis is a minute trichostrongyloid of the gastric mucosa mainly of cats. Females are ovoviviparous and lay third-stage larvae and transmission is by emesis, the larvae being passed in the vomit (Cameron, 1927). Because third-stage larvae are deposited in the stomach by the female worms, autoinfection probably occurs but it is not known what factors would control population levels in the final host.

Arrested development is an interesting and well-studied phenomenon of many trichostrongyloids (Michel, 1974; Schad, 1977). The infecting larvae enter the mucosa, attain the early fourth stage, and remain there for varying periods of time. Their emergence into the lumen of the gut and their reproduction there is coincident with external conditions suitable for transmission. It is extremely important in the survival of parasites in areas where there are prolonged periods unsuitable for the development of eggs or larvae in the external environment. The phenomenon has been intensively studied in livestock but it is known to occur in various wild animals including lagomorphs and deer (Baker and Anderson, 1974; Gibbs et al., 1977; Measures and Anderson, 1983).

Among the Strongyloidea the syngamids, including the gape worms of birds, make use of earthworms, terrestrial gastropods, and other invertebrates as paratenic hosts (Wehr, 1971).

In some hookworms (ancylostomatoids), larvae may invade the resistant mother cutaneously or orally and pass across the placenta to the fetus (Clapham, 1962). The worms only mature after the young are born. In addition, larvae may be present in the milk of the mother and be passed to the young while they are suckling.

Transmammary transmission, which is probably incipient in many hookworms of terrestrial mammals (e.g., Stone and Girardeau, 1966), has assumed great significance in *Uncinaria lucasi* of the northern fur seal (*Callorhinus ursinus*), which has made use of this characteristic to adapt to a host that spends 8 mo at sea (Olsen, 1958). Pups acquire infections from the milk of the mother and are the only members of the population on the rookery to have the adult worms. Eggs passed

by the pups contaminate the rookery and develop into third-stage larvae that hatch after the seals have left. When seals return 8 mo later new larvae penetrate their skin and accumulate in blubber and milk.

The metastrongyloids or lungworms of mammals, unlike the other bursate superfamilies, are essentially heteroxenous (Anderson, 1982). *Metastrongylus* spp. of swine use earthworms as intermediate hosts but most other species studied use terrestrial gastropods.

Heteroxeny within lungworms is of 3 types:

1) The intermediate host is an essential item in the diet of the final host. This is probably the primitive mode of transmission.
2) The intermediate host is accidentally ingested with the food of the final host. This type of transmission depends upon the host consuming large quantities of ground vegetation and is found mainly among the lungworms of ruminants and lagomorphs.
3) A vertebrate paratenic host is placed between the intermediate host and the final host. The paratenic host feeds on gastropods and is itself a consistent part of the diet of the final host.

Paratenesis is undoubtedly a common feature of the transmission of lungworms of carnivores and its evolution would have arisen in response to shifts in food preferences of the final host from molluscs to prey vertebrates, which themselves continued to consume gastropods consistently. It would have been crucial for the survival of many groups of lungworms during the radiation of carnivores from unspecialized ancestors (Anderson, 1982).

Filaroides decorata of the California sea lion (*Callorhinus ursinus*) develops to the third stage in coprophagous fish, which are then eaten by the final host (Dailey, 1970), raising the possibility that this parasite evolved from forms that used fish paratenic hosts and that the paratenic host has, during the course of evolution, become an intermediate host.

Filaroides osleri and *F. hirthi* of dogs are transmitted directly by means of first-stage larvae during regurgitative feeding (Dorrington, 1968; Georgi, 1976; Dunsmore and Spratt, 1979). Such a transmission is most unexpected in the lungworms but the evidence indicates that *F. osleri* behaves similarly in wild canids.

In *Protostrongylus* spp. of bighorn sheep (*Ovis canadensis*) the infective larvae from alpine snails ingested by the pregnant ewe cross the placenta and lodge in the liver of the fetus (Hibler et al., 1972). They move to the lungs and mature only after birth of the lamb.

Oxyuroidea

Oxyuroids are highly developed in insects and it is assumed that forms in vertebrates are derived from those in insects (Chitwood, 1940). The human pin worm is the best known oxyuroid in vertebrates but oxyuroids are common in wild terrestrial animals, especially those with large caeca and which are coprophagic as, for example, lagomorphs and rodents.

In oxyuroids there are generally 2 molts in the egg which is then infective to the host. Transmission is, therefore, direct and contaminative.

Oxyuroids are rare in aquatic hosts probably because monoxeny is not a successful life style for parasites in aquatic habitats. *Gyrinicola batrachiensis* of tadpoles of anurans is of interest, not only because it is one of the rare oxyuroids in an aquatic host, but because the ventral horn of the uterus produces thin-shelled eggs that are autoinfective and the dorsal horn produces thick-shelled eggs that are passed into the external environment and are the transmission stage (Adamson, 1981). In spring, thick-shelled eggs predominate when young-of-the-year tadpoles make their appearance. The parasites disappear from tadpoles just prior to the eruption of the forelimbs and frogs cannot be infected experimentally.

Ascaridida

The Ascaridida includes 5 morphologically related superfamilies with diverse patterns of transmission.

Cosmocercoidea: The cosmocercoids are common parasites of the gut of amphibians and reptiles and in some species the third stage is attained in the external environment after which it penetrates the skin of the host (Baker, 1978). Other species might be transmitted orally but it is not known if an intermediate or paratenic host is involved (Barus and Groschaft, 1962; Bartlett and Anderson, 1985). Some cosmocercoids have morphologic affinities to ascaridoids but the transmission of some of them is similar to that of unspecialized trichostrongyloids. The ascaridoids may have evolved from cosmocercoid-like ancestors (Chabaud, 1957).

Cosmocercoides dukae of the respiratory chamber of terrestrial gastropods is an example

of a parasite that has transferred from amphibians to an entirely different phylum where it has speciated (Vanderburg and Anderson, 1987). This parasite can be transmitted transovarially (Anderson, 1960).

The members of the Atractidae are unusual in that some multiply endogenously in the host and have been able to infect animals not readily susceptible to infection by a percutaneous route (Baker, 1982).

Heterakoidea: The Heterakoidea includes *Heterakis* of *Histomonas* fame. There is a molt in the egg and second-stage larvae may be harbored by earthworms (Wehr, 1971). In the bird host there may be a brief tissue phase where the third stage is attained before development to the adult stage occurs but this has not been definitely established.

Subuluroidea: In subuluroids the egg contains a first-stage larva that develops in insects to the third stage (Quentin, 1969b; Quentin and Tchéprakoff, 1969; Seureau and Quentin, 1986). The development of subuluroids is similar to that found in the unrelated Spirurida and the similarities are probably a good example of convergence in transmission strategies.

Seuratoidea: The transmission of members of the Seuratoidea has not been extensively studied. However, species of some genera such as *Seuratum* utilize insect intermediate hosts and combined with their morphology suggest a link between the superfamily and the Spirurida (Quentin, 1970a).

Truttaedacnitis stelmioides in brook lamprey (*Lampetra lamottei*) develops to the infective stage in the tissues of the ammocoete, which has a life span of 4 yr (Pybus et al., 1978). It is only when the ammocoete transforms that the larvae invade the gut and mature. Thus, the ammocoete acts as an intermediate host for a parasite of the adult. Other seuratoids in the family Cucullanidae may develop as larvae in immature fish and only proceed to the adult stage in the mature final host.

Ascaridoidea: Transmission within the ascaridoids is unusually varied and there is a dearth of epizootiological information which would help to relate transmission and development to the behavior of hosts. It has been proposed (Anderson, 1984) that the earliest intermediate hosts were vertebrates and that the simple 3-host system is primitive. In this system there is a molt in the egg to the second stage. The latter is ingested by an invertebrate paratenic host that transfers the larva to a vertebrate intermediate host in which development occurs to the *early third stage* which is infective to the final host. This system is commonly found among ascaridoids, including *Contracaecum* spp. (Huizinga, 1966, 1967), and it can be used to derive other strategies displayed in the transmission of ascaridoids. Most importantly in this system it is the early third stage that is infective to the final host and this is regarded as a primitive feature in all the secernentean parasites of vertebrate animals. Adamson (1986) has pointed out that this hypothesis helps explain the extreme plasticity of the group since in many instances "... intermediate and final hosts are physiologically quite similar."

Two molts are reported in the eggs of some species (Araujo, 1971; Berry and Cannon, 1981; Smith et al., 1983) but these are regarded as specializations.

In certain species the paratenic host is lost and the eggs containing second-stage larvae are directly infective to the vertebrate intermediate host as in *Baylisascaris devosi* (see Sprent, 1953). In species of the well-studied genera *Ascaris* and *Toxocara* the vertebrate intermediate host is replaced by a tissue migration and development in the final host (for reviews see Sprent, 1962; Beaver, 1969). There is evidence that *Hysterothylacium analarum* of sunfish (*Lepomis gibbosus*) has a tissue phase after which it invades the gut and matures (Rye, 1987). Thus, the same pattern is found in some ascaridoids of both terrestrial and freshwater hosts.

In marine *Anisakis* spp. and *Hysterothylacium* spp. there is considerable evidence that development takes place in the invertebrate paratenic host as well as in the fish prey host, which is regarded herein as the primitive intermediate host (Oshima, 1972; Smith and Wooten, 1978; Deardorff and Overstreet, 1981a, 1981b). This leads to the possibility of eliminating the vertebrate intermediate host and replacing it with what was the paratenic host. This has, according to our scheme apparently occurred in *Porrocaecum ensicaudatum* of passeriform birds which develops to the third stage in oligochaetes (Osche, 1955) and also in *Sulcascaris sulcata* of marine turtles which reaches the fourth stage in marine molluscs (Berry and Cannon, 1981).

Precocity in intermediate hosts is evidently an important feature of the transmission of many ascaridoids. It is found in its simplest form in *Raphidascaris acus* of pike (*Esox lucius*) in which

the second-stage larvae develop to the fourth stage in the liver of fish intermediate hosts and are then infective to the final host (Smith, 1984). This precocious development in the intermediate host presumably accelerates the rate of development of the parasite to adulthood in the final host, an advantage to ascaridoids with seasonal transmission as in *R. acus* (see Smith, 1986).

Precocity may also manifest itself not only in the vertebrate intermediate host but also in what was primitively the invertebrate paratenic host. This would explain the common presence of fourth-stage larvae of species of *Anisakis* and *Hysterothylacium* in a variety of marine invertebrates as well as in fish; the final hosts of these species are carnivorous fishes and marine mammals (Oshima, 1972; Deardorff and Overstreet, 1981a, 1981b).

Precocity may even lead to larvae developing to the adult stage in the vertebrate intermediate host. This may account for the presence of adult *Hysterothylacium haze* in the body cavity and tissues of yellowfin goby (*Acanthogobius flavimanus*) in Japan (Machida et al., 1978). Similar precocity has been reported in *Hexametra angusticaecoides* of chameleons (intermediate host) and boas (final host) and in *Orneoascaris chrysanthemoides* of frogs (intermediate host) and night adders (final host) (Sprent, 1982).

Precocity of this extreme type would almost totally eliminate the time for worms to reach maturity in the final host and it may have evolved as a transmission strategy. In some cases, extreme precocity might have allowed ascaridoids to adopt an independent existence in what was an intermediate host assuming there is some way eggs could get to the external environment, e.g., by the adult worms occupying the gut. Thus, precocity might be a mechanism by which ascaridoids have sometimes transferred from predators to prey.

Sprent (1982) has proposed an alternative hypothesis, namely that certain ascaridoids transferred from prey hosts to predators followed by "... suppression of the egg laying stage in the prey host, so that the prey became an intermediate host." This hypothesis has the complication that not only would egg laying have to be suppressed in the prey host, but the fourth and/or third molts would also have to be suppressed.

Spirurida

The Spirurida is a large and complex order of nematodes that produce eggs containing a first-stage larva and use arthropods as intermediate hosts. Transmission often occurs through the medium of water with some species developing to the third stage in copepods or aquatic crustaceans and others using aquatic stages of insects. Species in terrestrial hosts use terrestrial insects or crustaceans. Seureau and Quentin (1986) have reviewed the development of numerous species of the Spirurida in locusts (*Locusta migratoria*). Often, however, different species within the same genus will utilize widely different intermediate hosts depending on the biology of the final host. Thus the type of intermediate host is, to a certain extent, ecologically determined.

Since arthropods are such important dietary items for many animals, it is not unexpected that transmission of many spirurids takes place when the final host deliberately eats the intermediate host. However, some spirurids of ungulates reach the host when it accidently ingests arthropods while grazing. In addition, some species are known to use paratenic hosts.

The use of vectors has greatly expanded the host range of some groups of spirurids in that it has made transmission independent of the food habits of the final host. The important group of the Filarioidea has developed the microfilaria and adopted a wide range of hematophagous insects and acarines as intermediate hosts and vectors.

The Spirurida is divided into the Camallanina with the superfamilies Camallanoidea and Dracunculoidea and the Spirurina with the remaining superfamilies.

Camallanoidea, Dracunculoidea, and Gnathostomatoidea: Members of these 3 superfamilies use copepod intermediate hosts. First-stage larvae are highly active in water and attractive to copepods.

Camallanoids are parasites mainly of fishes, frogs, and turtles. Smaller fish can acquire infective larvae from ingesting copepods but in some species larvae can transfer from small forage fish to larger predatory species (Moravec, 1971; Stromberg and Crites, 1974). According to Moorthy (1938) there is some development in these small fishes serving as paratenic hosts and Pereira et al. (1936) believed that development to the fourth stage occurred. Crites (1976) obtained adult worms from fish experimentally infected with encapsulated fourth-stage larvae of *Camallanus oxycephalus* from drum (*Aplodinotus grunniens*). Thus, paratenesis and precocity are probably significant features of the trans-

mission of the camallanoids, including those in fishes and it may help to explain the very wide host distribution of many species. Camallanoids (e.g., *Serpenema trispinosus*) of turtles are probably transmitted through the agency of fish and invertebrate paratenic hosts such as snails (Bartlett and Anderson, 1985). Overstreet (1978) reported larvae of *Spirocamallanus cricotus* of marine fish in squid (*Loliguncula brevis*).

The Dracunculoidea has representatives in fishes, birds, amphibians, reptiles, and mammals. In most species the inseminated female migrates from the peritoneal cavity to subcutaneous tissues, especially of the appendages of the host, and releases active first-stage larvae into water through a skin lesion; males die after inseminating females. In some species in fishes, however, females become gravid in the peritoneal cavity and larvae pass out with the reproductive products of the host (Platzer and Adams, 1967; Ko and Adams, 1969; Lewis et al., 1974). Transmission of dracunculoids of fishes is generally through ingestion of the intermediate host and in wild hosts is often coincident with blooms of copepods (Uhazy, 1977a, 1977b).

Brackett (1938) demonstrated transmission of *Dracunculus ophidensis* to snakes by means of tadpole paratenic hosts and Crichton (1972) noted that third-stage larvae of *Dracunculus insignis* of raccoons (*Procyon lotor*) would persist for prolonged periods in frogs (*Rana* sp.). One can probably assume that species of *Avioserpens* of fish-eating birds (e.g., herons) must be transmitted through fish or amphibian paratenic hosts.

Ichthyofilaria spp. in marine fish have a microfilarioid-like larva that is found in the blood of the host but their transmission has not been studied (Appy et al., 1985).

The Gnathostomatoidea contains 5 genera found in fishes, amphibians, reptiles, and mammals. *Spiroxys contortus* of turtles uses tadpoles, minnows (Hedrick, 1935), and snails (Bartlett and Anderson, 1985) as paratenic hosts in none of which the larvae seems to grow or develop. Species of *Gnathostoma* utilize a variety of fishes, amphibians, snakes, birds, and mammals as paratenic hosts (Miyazaki, 1960, 1962; Daengsvang, 1980) and humans acquire gnathostomiasis from eating paratenic hosts (especially freshwater fish); [illegible] be passed from one paratenic host to [illegible] though, larvae in paratenic hosts re[illegible] third stage, they grow considerably [illegible] precocity can be considered a feature of the transmission of the gnathostomes.

Ko et al. (1975) found third-stage larvae of *Echinocephalus sinensis* of the eagle ray (*Aetabatus flagellum*) in the Japanese oyster (*Crassostrea gigas*). Oysters are probably paratenic hosts as it would be unusual for a species of the Gnathostomatoidea to develop initially in anything other than arthropods, particularly copepods.

Rictularioidea: Species occur mainly in insectivores, bats, rodents, and carnivores and are known to reach the infective stage in terrestrial insects including field and camel crickets, cockroaches, and beetles (Quentin, 1969a; Quentin et al., 1976a). Paratenesis is probably a common feature of the transmission of rictularioids of carnivores. Larvae of *Pterygoderma cahirensis* of felids and canids have been reported encapsulated on the mesentery of reptiles; larvae reencapsulated in guinea pigs and were also infective to cats (Gupta and Pande, 1970).

Physalopteroidea: Physalopteroids occur in the stomach of all classes of vertebrates. Species of *Physaloptera* and *Turgida* of mammals develop to the infective stage in a variety of insects, especially orthopterans and coleopterans. Paratenesis is probably important in the transmission of species in some carnivores and snakes since infective larvae are known to attach to the stomach wall of frogs, snakes, and even small mammals and will persist in these hosts for prolonged periods (see, for example, Cawthorn and Anderson, 1976; Gray, 1981). Paratenesis in the physalopteroids is unusual in that larvae in the paratenic host occupy the same site as in the final host.

The transmission of *Skrjabinoptera phrynosoma* of the horned toad (*Phrynosoma cornutum*) seems to be adapted to arid conditions. Female worms are passed in the feces of the host. Eggs remain alive in capsules in the desiccated carcass of the female which is eaten by ants that serve as intermediate hosts (Lee, 1955, 1956).

Thelazioidea: The Thelaziidae includes the eye worms of the genera *Oxyspirura* and *Thelazia* of birds and mammals. Eggs of species of *Oxyspirura* pass down the lacrymal duct and out in the feces of the host and development occurs in cockroaches (Fielding, 1927). Species of *Thelazia*, on the other hand, deposit first-stage larvae into lacrymal secretions from which they are obtained by muscoid flies that serve as intermediate hosts and vectors (Krastin, 1949). Anderson (1957a) proposed that the life cycles of the filar-

ioids may have originated from those of thelaziid-like ancestors.

The Rhabdochonidae includes *Rhabdochona* spp. of fishes which develop in the larvae of aquatic insects (e.g., mayflies) and crustaceans. The development of certain species in the intermediate host is of exceptional interest because the worms sometimes attain the adult stage in these invertebrates (Gustavson, 1942; Poinar and Kannangara, 1972); at least one species of *Ascarophis* of the superfamily Spiruroidea (see below) is also known to attain the adult stage in amphipods (Appy and Butterworth, 1983).

Recent studies by P. J. B. Byrne (unpubl. data) have indicated the significance of extreme precocity in a species of *Rhabdochona* of the common shiner (*Notropis cornutus*). In this case, the fish always spawn in a specific region of the stream and remain there for only a few weeks during which time they acquire precocious adult worms found in the local mayfly larvae (*Ephemera simulans*). The worms are ready to produce eggs almost as soon as they reach the gut of the fish host. The fish excrete nematode eggs into the spawning area, reinfect the local mayfly population, and then disperse. Thus, precocity in this parasite is a strategy that accelerates maturation in the final host to make the best of the very limited time and space available for transmission. Other examples of precocity among the Spirurida may have a similar explanation.

Spiruroidea: Spiruroids occur mainly in terrestrial mammals and less commonly in terrestrial birds and reptiles. Chabaud (1965) and Quentin (1970b) have briefly reviewed the general biology of members of the Spiruroidea that have been investigated. Many species have been shown experimentally to develop in insects but there is a dearth of epizootiological information that would clarify transmission under natural conditions. Transmission seems to be fairly uniform in the superfamily, however. Eggs containing first-stage larvae hatch in various kinds of insects, in which they develop to the early third stage which is infective to the definite host. Intermediate hosts are ingested accidentally (e.g., *Gongylonema* spp. in ungulates) or deliberately. Species in carnivores or omnivores (e.g., *Spirocerca lupi* of canids; *Ascarops strongylina* and *Physocephalus sexalatus* of swine) also make use of vertebrate paratenic hosts (Krahwinkel and McCue, 1967).

Habronematoidea: The superfamily is large and complex with species occurring in all vertebrate classes. Some species in terrestrial hosts are transmitted in the usual way through terrestrial insects (especially orthopterans) (Seureau and Quentin, 1983; Bartlett et al., 1984). Species in fishes develop to the infective stage in insects (e.g., mayflies) and especially amphipods, isopods, and mysids (see, for example, Keppner, 1975; Smith and Lankester, 1979; Jilek and Crites, 1982; Appy and Dadswell, 1983). *Hedruris androphora* of amphibians and reptiles is of interest because Leuckart (1876, p. 545) reported that it attained the adult stage in isopods; this was the first report of extreme precocity in the Nematoda.

Species of *Habronema, Parabronema,* and *Draschia* of the gut of cattle and horses develop in larval muscoids found in feces. The infective larvae move to the mouth parts of the adult fly and are transmitted when the latter feeds about the nares and mouth of the definite host. Chandler et al. (1941) summarized the life cycles of species of *Habronema* and *Draschia* and suggested that this type of transmission was the forerunner of that of the filarioids (cf. Anderson, 1957a).

Acuarioidea: The acuarioids constitute a fairly homogeneous group of nematodes found mainly in the upper digestive tract of birds and much less commonly in the stomach of small mammals. Species in strictly terrestrial birds (e.g., Galliformes and Passeriformes) develop in terrestrial insects and crustaceans (Cram, 1931; Wehr, 1971; Birova et al., 1974). Species in piscivorous birds develop in ostracods and amphipods and also make use of fish and frog paratenic hosts (Chabaud, 1950; Anderson and Wong, 1982; Wong and Anderson, 1982, 1987). Acuarioids in nonpiscivorous ducks develop in cladocerans (Austin and Welch, 1972). Species in shore birds (Charadriiformes) use amphipod intermediate hosts, including those in marine habitats (Wong and Anderson, unpubl. data).

In colonial piscivorous birds like gulls and cormorants, transmission occurs mainly on the breeding grounds and young birds acquire infections from fish paratenic hosts soon after hatching. Waders that breed in the Arctic acquire their infections on staging and wintering areas where the birds are highly concentrated and feed on marine crustaceans (Wong and Anderson, unpubl. data).

Aproctoidea and Diplotriaenoidea: Aproctoids occur in the air sacs, orbits, and nasal passages of birds. Eggs are passed in the feces of the

host. There are 2 families. Members of the Aproctidae are found mainly in passeriforms and *Aprocta cylindrica* develops to the infective stage in locusts (Quentin et al., 1976b). Members of the Desmidocercidae occur in the air sacs of piscivorous birds (e.g., herons and cormorants) and there is evidence that fish serve as paratenic hosts (Dubinin, 1949) but their transmission is little understood.

The diplotriaenoids inhabit the air sacs of birds and reptiles, and species of one genus, *Diplotriaena*, are common in many types of insectivorous birds. Eggs are passed in feces of the host. Larvae of *Diplotriaenia* and *Serratospiculum* develop in grasshoppers and locusts (Anderson, 1957b, 1962; Bain and Vassiliades, 1969; Bain and Vaucher, 1973; Cawthorn and Anderson, 1980).

Filarioidea: By freeing themselves from the food chain of the definitive host and developing the microfilaria, filarioids have been able to radiate extensively throughout the tissues and in all the vertebrate classes other than fishes. The literature is extensive. The Filariidae includes the more primitive species associated with skin lesions. Species of *Parafilaria* in horses and cattle release eggs through a break in the skin resulting in "summer bleeding" and muscoid flies feeding on the blood serve as intermediate hosts and vectors (Gnedina and Osipov, 1960). Species of *Stephanofilaria* are associated with a dermatitis attractive to horn flies which acquire larvae while feeding on the lesion and serve as intermediate hosts and vectors (Ivashkin et al., 1963; Hibler, 1966). Anderson (1957a, 1958) suggested that transmission involving skin lesions elicited by the nematode in the host was the forerunner of transmission involving microfilariae in blood or skin (i.e., Onchocercidae).

In species of the Onchocercidae, microfilariae occur in the skin or in the blood depending on the species. They are transmitted by a wide range of hematophagous insects and acarines (Schacher, 1973). Different species in the same genus may be transmitted by widely different vectors as for example those in the genus *Pelecitus*, which are transmitted by mosquitoes, tabanids, or mallophagans depending on the species (Bartlett and Anderson, 1988).

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TREMATODE TRANSMISSION PATTERNS

Wesley L. Shoop
Merck Institute for Therapeutic Research, Rahway, New Jersey 07065-0900

ABSTRACT: The primitive transmission pattern in the Trematoda is a 1-host pattern in which an aspidocotylean matures in a mollusc. The progeny from the parent aspidocotylean typically disseminate from the mollusc to establish in others, but the possibility that some progeny remain in the same mollusc as did the parent and produce a sequential generation exists. It is this potential for sequential generations in the mollusc that may be the key to understanding the complex transmission and life cycle patterns in the Digenea.

In this analysis, the patterns of transmission within the Trematoda are presented in 2 parts. Part I is a broad, generalized introduction into the basic transmission patterns as I perceive them. The patterns are placed in an evolutionary context and current thinking concerning each is discussed. In the second part, I add to the basic patterns the newest and most extraordinary mode of transmission to be elucidated within the Trematoda, transmammary transmission, and show that it represents the zenith of complex transmission and life cycle patterns in this group of helminths.

BASIC PATTERNS

In Figure 1 we observe the basic transmission patterns in the digenetic trematodes. Nearly ubiquitous to the trematode life cycle is the presence of a molluscan first intermediate host (with very rare exceptions annelids serve as first intermediate hosts). It is interesting that of the scores of partial and complete life cycles elucidated only 2 generalized transmission routes to this ancient host have been discovered. The most primitive route is through direct penetration of the molluscan epithelium by a free-swimming, ciliated larva known as a miracidium. Upon entry into the mollusc, the miracidium sheds it ciliated coat, transforms into a germinal sac, and undergoes a series of complex multiplicative events culminating with the mass production of cercariae. The second pathway is but a slight variation of the first. Here the miracidium remains in the egg and is passively ingested by the mollusc. Within the molluscan GI tract the miracidium then hatches, penetrates the endothelial lining, transforms into a germinal sac, and produces cercariae in mass quantities similar to that alluded to before.

Even though both of these transmission routes to the mollusc are quite similar, each could be interpreted as emphasizing a different strategy (Pearson, 1972; Overstreet, 1978). The first pathway entails the production of a small number of large eggs. The resulting miracidia exit the eggs and survive for only brief periods (up to 48 hr) whereupon they must find and penetrate an appropriate mollusc or die. This mobile strategy represents dissemination of the parasite in space. In the second pathway, there is production of many small eggs that do not hatch. The miracidia instead remain within the eggs for extended periods of time and await ingestion by the mollusc. The ability to mass produce small eggs overcomes the great loss of those either never ingested or ingested by unsuitable hosts. This sedentary strategy represents dissemination of the parasite in time.

Once the mollusc has been colonized, it is usually the cercarial generation developing within that characterizes the innovations leading to the exotic life cycle patterns of digenetic trematodes. The simplest and most primitive pattern of cercarial transmission in the Digenea occurs when the mollusc, with unencysted cercariae, is ingested by a vertebrate definitive host (Fig. 1, Pattern 1). This transmission pattern is observed in a single monotypic family, the Heronimidae. In the life cycle of members of the genus *Heronimus* (Fig. 2), a vertebrate (reptile) ingests the molluscan intermediate host along with the unencysted cercariae contained in a mother sporocyst (or germinal sac). The mother sporocyst is a metamorphosed miracidium in which the germinal cells have fragmented and each newly formed germinal center produces a cercaria. When the cercariae are freed by the digestive properties of the vertebrate, maturation occurs in an embryological derivative of the GI tract (in this case the lungs). Subsequently, miracidia are disseminated by the vagile vertebrate and upon penetration of an appropriate mollusc the life cycle continues.

To understand why this fundamental 2-host pattern is considered the primitive pattern in the digenetic trematodes requires that we know how

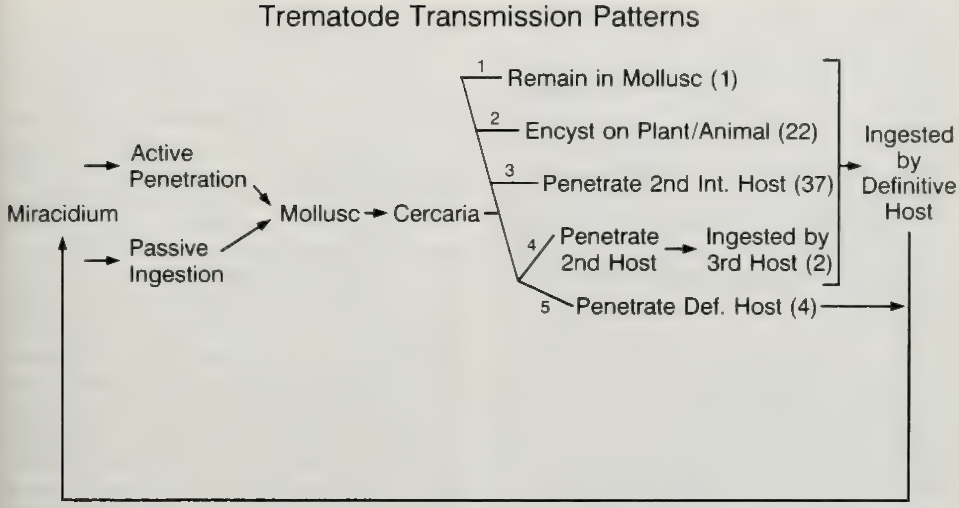


FIGURE 1. Five basic transmission patterns of digenetic trematodes as delineated by cercarial behavior. The numbers in parentheses represent the approximate number of families in which each pattern occurs.

it evolved. This entails looking for a moment outside the Digenea. As intimated by Rohde (1972) and Gibson (1981), and formalized by Brooks et al. (1985a), the sister group to the digenetic trematodes is a small obscure taxon of trematodes, the Aspidocotylea. This is enlightening because within the aspidocotyleans are 2 compelling life cycle patterns. The simplest of the 2 patterns is an obligate 1-host life cycle that involves the aspidocotylean in a parasitic association with a mollusc. The aspidocotylean matures to adulthood in the mollusc and produces a cotylocidium that is infective for that or other molluscs. The *potential* for the cotylocidium to remain in the mollusc and produce an additional generation will become important, as I discuss later, in understanding the origins of the intramolluscan generations of the Digenea.

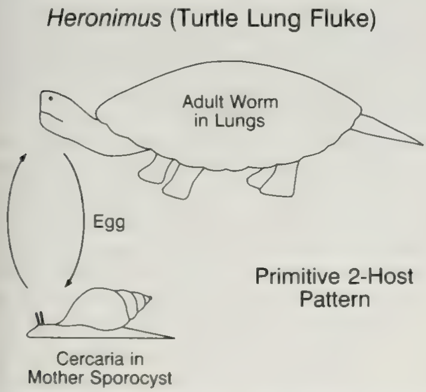


FIGURE 2. The primitive 2-host pattern of the genus *Heronimus*.

The second pattern is a derived 2-host life cycle in which the aspidocotylean is in a parasitic association with both a mollusc and a vertebrate (fish or reptile). Development within the mollusc is extensive and often includes sexual maturation. The vertebrate acquires the parasite by ingesting the mollusc directly and may or may not play an essential role in the life cycle. The aspidocotylean survives ingestion and deposits eggs within the GI tract of the vertebrate predator. Dissemination of the parasite progeny far and wide by the vagile vertebrate has obvious advantages. Significantly though, the disseminated cotylocidia are infective only for other molluscs.

It appears then that derived aspidocotyleans and the heronimids have comparable life cycle patterns with comparable patterns of transmission. The observations on the Aspidocotylea represent more than a superficial relationship to the 2-host heronimid cycle because Brooks et al. (1985b) made the significant discovery that the most primitive family within the Digenea, and thus that closest to the Aspidocotylea, is the Heronimidae.

Consequently, we infer from the linkage of the Aspidocotylea and Digenea that the original host

to all trematodes was the mollusc and that at one time maturity and sexual reproduction took place within it. At a later date, probably corresponding to the evolution of primitive vertebrates, the mollusc and its symbiont fell prey to bottom-feeding fishes and reptiles. The ability of some trematodes to survive in the vertebrate GI tract signaled the beginning of a 2-host life cycle. Therefore, primitive transmission to the vertebrate occurred through ingestion of the trematode and the only free-living phase in this basic life cycle served to facilitate transmission back to the mollusc.

Although it is the much studied cercarial stage that is so obvious in digenean transmission patterns, it is to the other cryptic intramolluscan stages that we should turn to understand the evolution of these transmission patterns. It is my opinion that each intramolluscan "stage" of present-day digeneans is a modified version of the 1-host aspidocotylean cycle. In that 1-host pattern, the adult reproduces sexually and, through the production of cotylocidia, has the *potential* of giving rise to a second generation of adults that remain in the mollusc.

The intramolluscan events characteristic of the digeneans appear superficially to be quite different from the aspidocotyleans because of specialization and degeneration, but similarities are still discernible. The best cytological evidence suggests that reproduction in the digenean mother sporocyst includes a form of degenerate meiosis (Khalil and Cable, 1968). Degenerate or not, evidence of meiosis clearly indicates that the mother sporocyst is a vestigial adult and not a larva. This is, of course, concordant with the belief that the mollusc was the original host of all trematodes and that sexual maturity took place within it. Some mother sporocysts of present-day species may have lost all vestiges of meiosis and appear to be asexual, but this resulting asexual condition is certainly a derived state (James and Bowers, 1967). From an evolutionary perspective, if you accept that the mollusc was the original host of adult Trematoda, then asexual reproduction as a primitive condition makes no sense whatsoever. On theoretical grounds, the mother sporocyst is, then, a functional equivalent to the aspidocotylean adult. The fragmented oogonia in the body of the mother sporocyst represent the vestiges of an ovary. The oogonia divide and, through direct development, produce members of the next generation, i.e., either rediae, daughter sporocysts, or cercariae. Although we often refer to these as "stages," each is actually an equivalent generation that has a sexually reproducing adult or some vestige thereof.

The similarities of the aspidocotyleans to the digeneans are masked because in the former the generations are oviparous and morphologically identical, whereas in the latter they are viviparous and typified by highly modified morphotypes. The aspidocotylean life cycle is correctly characterized as a single generation from adult to egg and back to adult. However, in the so-called digenean "life cycle" the egg stage from each intramolluscan generation has atrophied and the generations condensed to such a point we hardly recognize them as distinct entities any longer. As an unfortunate result, we have, in my opinion, mistakenly fused multiple generations into what is called a single "life cycle." For instance, from a mother sporocyst to oogonia and back to a redia is a generation equivalent to the aspidocotylean; so is a redia to oogonia and back to cercaria. It is the way we have conceptualized these generations that has kept us from realizing how similar to aspidocotyleans they really are.

If the digenean generations are a multiplication of the aspidocotylean generation, then we must explain the evolution of viviparity, degeneracy, modified morphotypes, and vestigial sex. To begin, each of the protodigenean generations was undoubtedly capable of producing free-living transmission stages that left the mollusc, as observed in extant aspidocotyleans. However, with the advent of the 2-host pattern, when the vertebrate began to serve as a disseminator, the tremendous spatial dissemination afforded the progeny of the last intramolluscan generation would have rendered the brief and limited disseminative phase of any previous generation inconsequential. Those trematodes able to take advantage of the vertebrate disseminator would easily have been more effective at transmitting to and colonizing other molluscs than those that relied on their own limited vagility. As the success of those utilizing this method of wide dissemination grew, the dependence upon the vertebrate became fixed, and the early generation's capacity to produce its own disseminative stages diminished. Oviparity, required for the protection of the disseminated stages, was subsequently lost to viviparity in all but the last generation. The last generation, whose maturation in the vertebrate had become obligatory, maintained oviparity for dissemination back to the mollusc.

Having lost its own disseminative stages and

with transmission to the vertebrate emerging as the exclusive domain of the cercaria/adult generation, the fate of the ancestral mother sporocyst was sealed. Rapid and massive production of cercariae became necessary prior to the mollusc being consumed. Therefore, ontogenetic development streamlined and the ancestral mother sporocyst degenerated to serve as an incubator (germinal sac) for cercarial production. At this point, each of the 2 generations diverged morphologically as they specialized for their respective roles in the mollusc and vertebrate.

After the vertebrate had become part of the pattern, and concurrent with loss of disseminative stages and oviparity, came the suppression of sexuality in the first intramolluscan generation. Why this occurred is part of a great biological debate over the "costs of sexual reproduction." Indeed, many have wondered what with the 2-fold genetic advantage of parthenogens and asexual forms why there is sexuality at all (Williams, 1977; Maynard-Smith, 1986). The consensus of opinion would suggest that sex is associated with the production of genetically diverse propagules disseminated in the face of an uncertain future. Clearly, as the progeny of the miracidium/mother sporocyst generation lost the capacity to disseminate and remained in the mollusc, the genetic costs of their sexual production were no longer tolerable. This is reinforced further by the fact that to the progeny of the mother sporocyst, the already colonized intramolluscan environment was a known and relatively stable environment. Simply stated for the first digenean intramolluscan generation, the advantages of sex were no longer needed, the genetic costs were too high to be maintained and it is in the process of being lost. Therefore, we would expect to find extant trematode species at various steps in transition from full-blown sexuality, through meiotic and ameiotic parthenogenesis, to asexual budding. This is precisely what has been observed. Of course, one has to look at both the Aspidocotylea and the Digenea for the entire breadth of the spectrum.

This "loss of sex" scenario is not relevant to the generation that matured in the vertebrate. Because the propagules leaving the adult in that generation must be disseminated in what clearly is an unpredictable environment, sexuality along with oviparity has been maintained.

Consequently, the events occurring in the Digenea can be described by analogy to island biogeography. That is, a single colonizing propagule (miracidium) makes it to an island (mollusc), matures (mother sporocyst), and is able to reproduce (aided by loss of sex). It then runs several generations before the carrying capacity of the island is exhausted and disseminative propagules (cercariae) are produced. However, there is an important point to be made at this juncture in the analogy. In the typical digenean, the disseminative propagule from the mollusc would be predicted to be sexually produced, but cercariae are produced by mechanisms which appear to be losing, and in some cases have completely lost, their sexuality.

I believe this can only be explained by viewing a transmission pattern such as that in *Heronimus* as the most primitive condition. Therein, the cercariae are not disseminative stages at all for they remain in the mollusc until eaten by a vertebrate. No precarious dissemination to the vertebrate actually occurs and the advantages of sex are not required. To return to the island analogy, this is as if a land bridge arose between the original island (mollusc) and the new island (vertebrate) and no uncertain dissemination across hostile open water was necessary. By the time patterns evolved in which cercariae emerged from molluscs, their sexual production must have been irretrievably lost. Otherwise, the pattern of sexuality in the digenean generations makes no evolutionary sense. If other patterns in which cercariae emerge from the mollusc and seek out the next host were the primitive condition we should expect to find the cercariae still produced by sexual means. This, however, is not the case. Therefore, loss of sexuality in the intramolluscan generations of extant digeneans has to be a consequence of a 2-host, *Heronimus*-like heritage.

Heronimus represents the primitive and most simplistic condition in the Digenea in that only a single intramolluscan generation is produced, i.e., the metamorphosed miracidium (mother sporocyst) produces a single generation of immature adults (cercariae) that await ingestion by vertebrates. No redia/daughter sporocyst generation is known. The vertebrate hosts occurring in this pattern are primitive bottom-feeding vertebrates which ingest the mollusc directly. As stated, no high-risk disseminative form between mollusc and vertebrate is necessary. However, the evolution of the remainder of the Digenea as we know it is characterized by the addition of a redia/daughter sporocyst generation to the mother sporocyst (Brooks et al., 1985b). It is my con-

tention that it was the additional reproductive effort of this new generation, culminating in massive numbers of cercaria, which was the key innovation supporting the trematode solution to the myriads of nonmollusc-feeding vertebrates that were to evolve.

It is important to note that all of the patterns that I will mention henceforth are derived from the fundamental 2-host transmissive pattern observed in *Heronimus.* So before others are considered it is worth restating the basics. First, a highly vagile, vertebrate host in which the digenetic trematode matures will almost always be encountered. Because transmission to the vertebrate is facilitated by ingestion, the site of maturation is invariably the GI tract and its embryological derivatives (salivary gland ducts, naso-lacrimal ducts, eustachian tubes, lungs, liver, pancreatic duct, and biliary tree). Eggs are typically voided in the excreta and disseminated by the vertebrate far and wide. Subsequently, a mollusc is infected by a miracidium and serves as a first intermediate host. Then through a series of parthenogenetic (asexual in derived cases) events within the mollusc there is production of the cercarial generation.

My ideas on the remaining patterns have been influenced to a great extent by the powerful writings of Pearson (1972) and Rohde (1972), which have been corroborated in large part recently by the phylogeny of the Trematoda as promulgated by Brooks et al. (1985b).

In Pattern 2 (Fig. 1) we visualize the trematode solution to the evolution of nonmollusc-feeding vertebrates. If cercariae are to establish in nonmollusc-feeding vertebrates, then leaving the mollusc is not an option, it is a necessity. In this pattern, the cercariae exit the molluscan host, assume a brief free-living existence, and ultimately encyst upon plants, animals, or inaminate objects that are food items of the vertebrate host. The natatorial habit of emergent cercariae disseminates them in space, and their encystment disseminates them in time. This is a marvelous behavioral response to facilitate transmission. It is also a very costly one. In support of this precarious form of transmission the monophyletic lineage from the heronimids leading to the rest of the digenetic trematodes is not only characterized, but can be defined, by the addition of a redia/daughter sporocyst generation to the mother sporocyst. The addition of this specialized generation provides a quantum increase in cercarial production over that provided by a single mother sporocyst and offsets the tremendous mortality suffered by the free-swimming pioneers. This is a common pattern of transmission in the Digenea observed in 22 families of the paramphistomiforms, echinostomatiforms, haploporiforms, and some hemiuriforms.

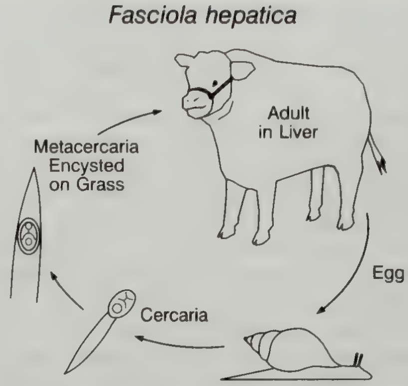


FIGURE 3. The 2-host pattern of *Fasciola hepatica* with a free-swimming cercaria encysting upon vegetation.

As an example of this pattern I have chosen *Fasciola hepatica* (Fig. 3). When most see differences, an evolutionist sees similarities. Therefore, the similarities to the primitive 2-host pattern will be emphasized first. There is a vertebrate disseminator (ruminant) with the parasite maturing in the GI tract or its derivatives (liver); the eggs are disseminated widely (on the pasture); a mollusc is infected; and multiplication in the mollusc culminates in mass production of cercariae. Now the innovations can be noted. Because cattle do not feed upon molluscs with any regularity, the cercaria, if it is to track the host, must intertwine itself in the host's food chain. Leaving the mollusc is requisite. In this example the cercaria has a brief free-swimming existence, then attaches to aquatic vegetation, pinches off the tail, forms a cyst wall, and goes into a hypobiotic state. When ruminants ingest food items bearing this encysted stage, the parasite promptly excysts in the GI tract, migrates to the biliary tree, and matures. The massive reproduction in the mollusc is a result of 2 parasite generations, i.e., the mother sporocyst giving rise to the rediae and then the rediae giving rise to the cercariae. The precarious dissemination of the cercariae could not be supported by the limited reproduction of the mother sporocyst alone. The encysted cercariae are known as metacercariae and they

Clonorchis sinensis

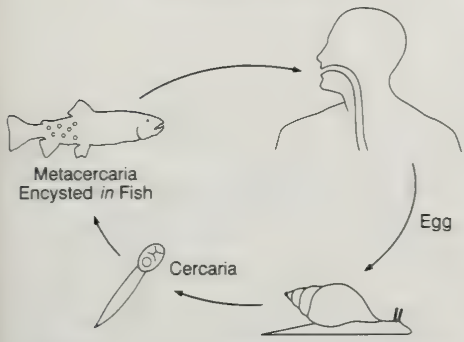


FIGURE 4. The 3-host pattern of *Clonorchis sinensis* with a free-swimming cercaria encysting within a second intermediate host.

foreshadow the derivation of the next life cycle pattern.

It is a short jump from encysting *upon* something to encysting *within* something. Pattern 3 (Fig. 1) is derived from Pattern 2 and is characterized by a cercaria penetrating a second intermediate host and encysting within it. Penetration of another host is not such a formidable leap in cercarial ability as might first appear. It should be kept in mind that early in its ontogeny the cercaria already possesses the ability to penetrate tissue as evidenced by its active escape from the mollusc. I infer from this that it also had the same capability in phylogeny. The penetration of a new host is a parasitic event and is significant because it represents the first time an obligate 3-host life cycle appears in the digenetic trematodes. The obligate 3-host pattern is by far the most common pattern in the Digenea and is found in 37 families (in some hemiuriforms, but more commonly in the strigeiform, opisthorchiiform, lepocreadiiform, and plagiorchiiform orders).

The life cycle of *Clonorchis sinensis* illustrates this pattern (Fig. 4). Again, the similarities to the previous pattern will be examined first. There is a vertebrate disseminator (human) with the parasite maturing in the GI tract or its derivatives (biliary tree); the eggs are disseminated widely; a mollusc is infected; a mother sporocyst gives rise to a generation of rediae which, in turn, produce a cercarial generation; and the cercariae escape the mollusc and become free-living. The innovations include the penetration of another host (fish second intermediate host) by the cercariae and their encystment under the epithelium. These behavioral adaptations are again supported by the massive intramolluscan reproduction that weaves the parasite into the food chain of a nonmollusc-feeding, yet carnivorous, vertebrate host.

Two additional patterns derived from the 3-host life cycle will be mentioned briefly. Specifically, the addition of a new host giving rise to a 4-host life cycle as well as the deletion of a host giving rise to another 2-host life cycle are outlined.

The 4-host life cycle (Fig. 1, Pattern 4) is quite rare in the digenetic trematodes and is found only in 2 strigeiform families, i.e., the diplostomids and the strigeids. In this pattern we find that a third intermediate host has been intercalated between the second intermediate and definitive host. The genus *Strigea* serves as a pertinent example of the obligate 4-host life cycle (Fig. 5). This cycle illustrates the fundamental vertebrate definitive host (carnivorous bird) in which maturation occurs in the GI tract; the eggs are disseminated widely; infection of a mollusc produces a mother sporocyst, daughter sporocyst generation, and cercarial generation; and the cercariae escape and penetrate a second intermediate host (amphibian). However, tadpoles are rarely food items of carnivorous birds such as hawks, owls, or herons, but they are the food items of aquatic snakes which, in turn, *are* food items of carnivorous birds. So in 2 rare instances in the Digenea we find the expansion of the 3-host life cycle to include the intercalation of a new host (third intermediate host) and a new life cycle stage (mesocercaria). The mesocercaria is clearly a result of paedomorphic heterochrony.

The derived 2-host life cycle (Fig. 1, Pattern 5) that will be illustrated is also an offshoot of the strigeiform lineage and is found in 3 families (spirorchids, sanguinicolids, and schistosomatids). This pattern appears superficially to violate the transmission patterns developed so far because the cercaria penetrates the definitive host directly. A typical life cycle illustrating this pattern is seen in *Heterobilharzia americana* (Fig. 6). This species is characterized by a vertebrate disseminator (mammal); eggs defecated in the mollusc's environment; subsequent infection of the mollusc; a mother sporocyst producing a generation of daughter sporocysts; and mass quantities of the cercarial generation emerging from the mollusc. However, at this juncture in the life cycle the cercaria penetrates the next host as if

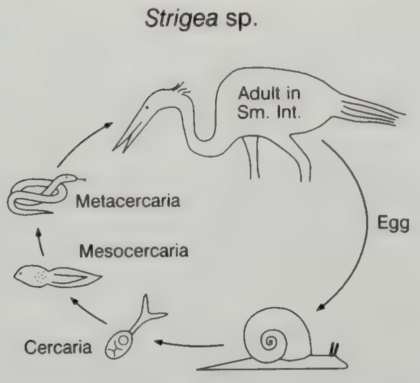


FIGURE 5. The 4-host pattern of the genus *Strigea* that resulted from the intercalation of a third intermediate host into the primitive 3-host pattern.

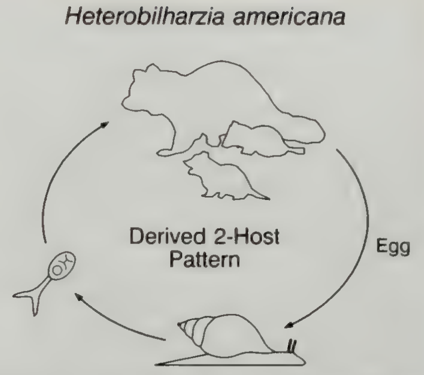


FIGURE 6. The 2-host pattern of *Heterobilharzia americana.*

it were a second intermediate host. The cercarial body (schistosomule) also migrates to the parenteral tissues (mesenteric venules) as if it were a second intermediate host, but maturation occurs as in a definitive host. How is this to be reconciled with the previous patterns?

Precocious (progenetic) development of metacercariae is known from, and has been an instrumental mechanism in abbreviating, numerous life cycles. Its terminal short-circuiting of life cycles may commence provided 2 requirements are met; (1) that the eggs have a route to the exterior, and (2) that the early release of the eggs is such that they come in proximity to the molluscan host. If either of these requirements is not satisfied, gametic wastage will be selected against. Assuming that blood fluke metacercariae experimented with precocious development at sometime in the past, there is a mechanism known for the eggs to reach the exterior. If you were fortunate enough to hear Dr. Ray Damian's Presidential Address last year in Denver the mechanism was provided (Damian, 1987). It is the inflammatory response of the host! The inflammatory response isolates foreign invaders such as bee stings and slivers and transports them to the exterior where they can be expelled. The schistosome eggs are also foreign invaders and when the cellular response expels them to the exterior, which in *H. americana* is the lumen of the intestine, there is a short circuiting of the 3-host life cycle and the second intermediate host becomes the definitive host. The original definitive host is rendered superfluous and is cleaved. This is why the "current" definitive host has so many second intermediate host characteristics. Making sense out of this pattern is possible only if one understands the 3-host pattern from which it was derived.

Precocious development has been instrumental in abbreviating other 3-host life cycles such as in the macroderoidid genus, *Alloglossidium.* For a lucid description of the evolution of this group and the significance of progenesis one should consult Font (1980) in the original.

Before I go on to the next part, allow me to recapitulate the evolution of the transmission and life cycle patterns. First, in the aspidocotyleans we have an obligate 1-host life cycle involving only a mollusc. Also within that group we see the acquisition of a 2-host life cycle when a vertebrate host ingests the mollusc. This is the primitive pattern seen in the digenetic trematodes and is illustrated by *Heronimus.* The potential for additional generations in the aspidocotyleans has been amplified in the digeneans. Whereas the aspidocotylean generations would be identical and repetitious, the digenean generations are condensed and typified by highly derived and degenerate morphotypes. Transmission to nonmollusc-feeding vertebrates necessitates the exiting of the cercarial stage from the mollusc and the intertwining of the parasite into the food web of the vertebrates. The precarious free-living existence of the emergent cercaria is supported by massive reproduction made possible by the addition of the redia/daughter sporocyst generation in the molluscan host. Encystment of the cercaria, hypobiosis, and transformation to the metacercaria are innovations that prolong the free-living phase. Consequently, acquisition of a second intermediate host occurred after the first intermediate and definitive host were firmly en-

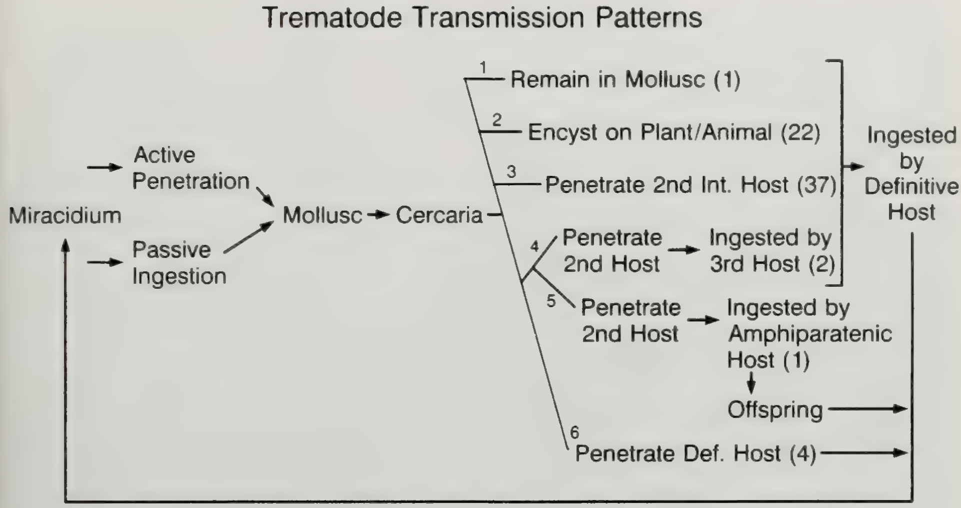


FIGURE 7. Vertical or transmammary transmission incorporated onto the original 5 patterns of Figure 1.

trenched in the digenetic trematode life cycle. This 3-host life cycle now represents the most common pattern in the Trematoda. As a by-product of this evolution, all forms of transmission in the trematodes can be ascribed to either ingestion or direct penetration.

THE NEWEST MODE OF TRANSMISSION: TRANSMAMMARY TRANSMISSION

And now I wish to discuss the most recently elucidated form of transmission in the trematodes, and one which represents an extraordinary innovation because it entails passage of the parasite in a second dimension—time. This transmission form is often called vertical or maternal transmission, or even more specifically to the case that I will discuss it can be referred to as diathelic, galactogenic, milk-borne, or transmammary transmission. What I hope to show is that this pattern of transmission in the Trematoda is derived from the 4-host life cycle (Fig. 7).

Historically, the first substantive evidence for transmammary transmission in a trematode was presented for *Pharyngostomoides procyonis* by Harris et al. (1967). Additional data were forthcoming when Miller (1981) reported the pattern of infection in offspring born to a host naturally infected with *Pharyngostomoides* spp. that left little doubt transmission via the milk had occurred. The insight these authors provided was extraordinary when considering that *Pharyngostomoides* spp. were not amenable to study in standard laboratory hosts and that virtually all of their observations came from captured, naturally infected animals. Controlled experimental evidence verifying their observations finally came when it was discovered that a closely related species, *Alaria marcianae*, also underwent transmammary transmission (Shoop and Corkum, 1983a). To date, most of what we know about milk-borne transmission in the Trematoda has come from studies on *Alaria marcianae* and this has been in large part due to the adaptability of that organism to laboratory animals.

Thus, I will use the *Alaria marcianae* life cycle to illustrate this newest pattern (Fig. 8). As evidenced in this flow chart there is a molluscan first intermediate host; intramolluscan development includes a mother sporocyst, daughter sporocyst generation, and cercarial generation; cercariae exit and directly penetrate an amphibian second intermediate host; mesocercariae develop; and if the tadpole is ingested by either a felid or canid definitive host, the worms mature in the GI tract. However, within the definitive host there is a very complex migration in which the ingested mesocercariae actually exit the GI tract, migrate to the lungs, and transform into metacercariae—as if this were a third intermediate host (Shoop and Corkum, 1983b). Then, à la Damian (1987), an inflammatory reaction expels the metacercariae from the lungs. The expulsion from the lungs is only the first of a series

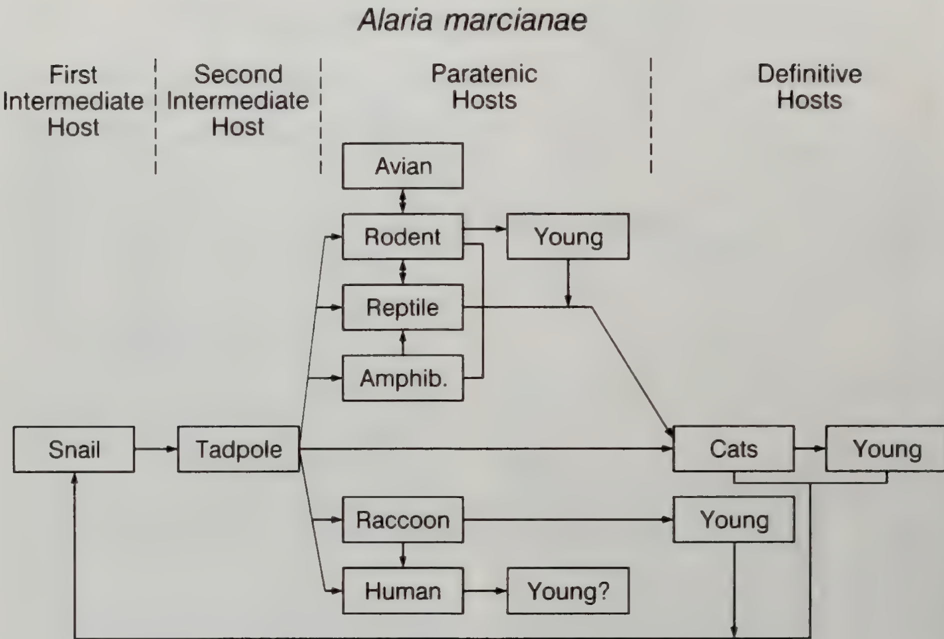
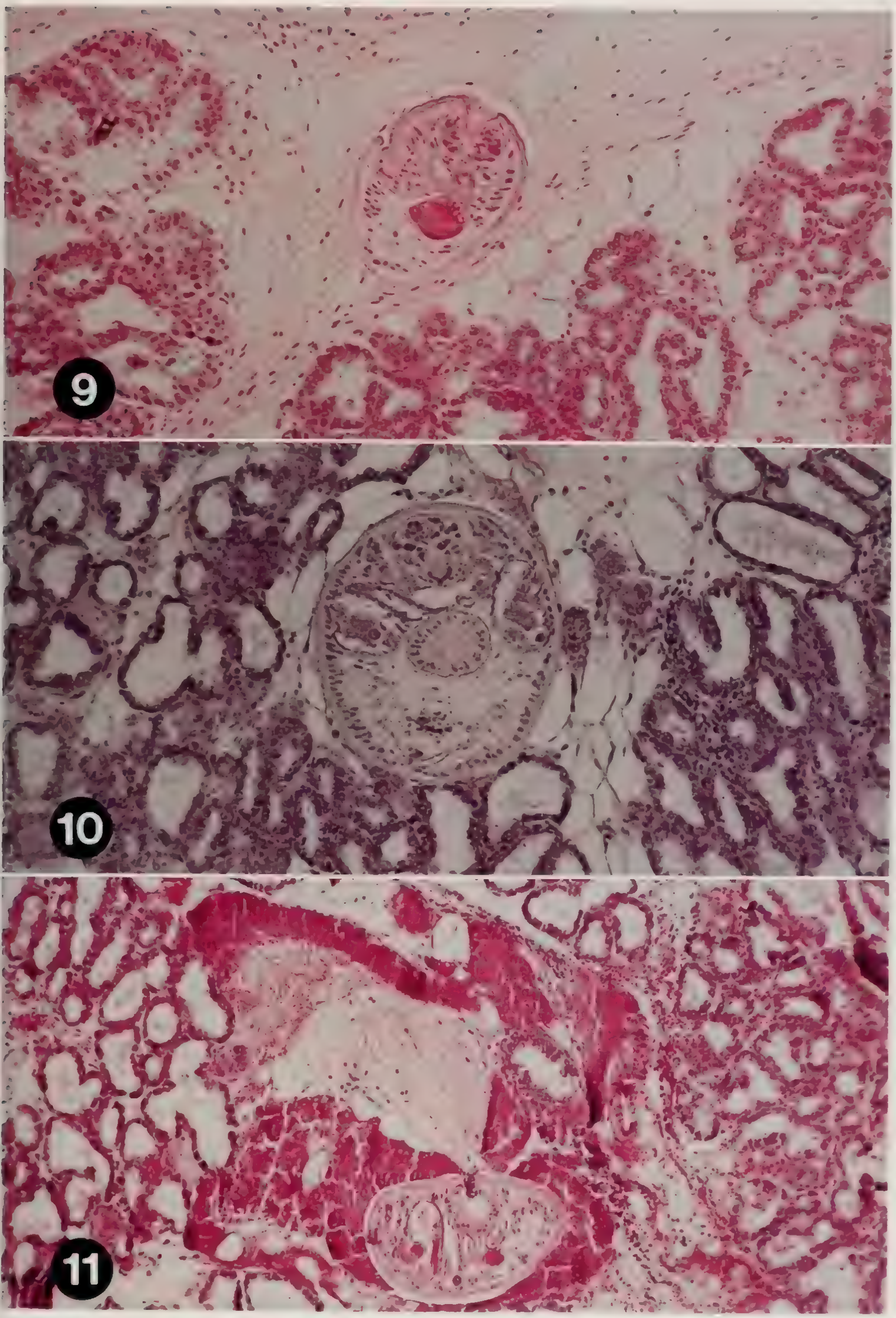


FIGURE 8. The 3-host pattern of *Alaria marcianae* and its paratenic relationships.

of host defense mechanisms designed to rid the body of the foreign invaders. As the metacercariae are exteriorized into bronchioles, cilia push them up the respiratory tree. Upon reaching the upper third of the trachea the cough reflex propels the worms into the buccal cavity and then the swallow reflex and peristalsis continue the attempt to purge the body of the trematodes.

It is interesting that this latter part of the expulsion also simulates the pathway the metacercariae would take if they were ingested while in a third intermediate host (as occurs in primitive members of the genus *Alaria*). When the metacercariae are carried to the small intestine they thwart the attempt at expulsion by attaching to villi where they then mature and reproduce. The host defense mechanisms appear to orchestrate this entire event because the metacercariae do not seem to be migratory stages at all (Shoop and Corkum, 1984b). Thus, we have a type of host that masquerades as both a third intermediate and definitive host, and what was originally a 4-host life cycle is now condensed into a 3-host life cycle. The original transmission event requiring the food web has acquiesced to the defense mechanisms for transmission.

The telescoping of the third intermediate and definitive host into one creates the attendant transmission difficulties of attempting to bridge the aquatic and terrestrial phases of the life cycle. These transmission difficulties have been overcome by innovations at the mesocercarial stage. For instance, when a tadpole infected with mesocercaria is ingested by a snake, the mesocercaria is transferred, but remains a mesocercaria; when the snake is ingested by an alligator, the mesocercaria is transferred again, but remains a mesocercaria; when the alligator dies and is scavenged by a rodent, the mesocercaria is transferred once more, but still remains a mesocercaria; and so it goes *ad infinitum*. This is a parasitological phenomenon described by Baer (1951), later refined by Beaver (1969), and is called paratenesis—a pattern of host-to-host transfer in which the parasite undergoes neither advancement in development nor reproduction. Embodied in this phenomenon is the ability of the parasite to persist in space and time until transport to the definitive host occurs. The mesocercaria does not initiate the ageing process by turning on the "old-age genes" until the right stimulus is provided, i.e., the lungs of the definitive host, and consequently it remains hypobiotic and is shuttled throughout the food web for years and years;



FIGURES 9–11. **9.** Mesocercaria of *Alaria marcianae* in the dense connective tissue surrounding a mouse lobule. **10.** Mesocercaria of *Alaria marcianae* in a track of lipocytes within a mouse lobule. **11.** Mesocercaria of *Alaria marcianae* in a pool of milk created from the destruction of an alveolar bed.

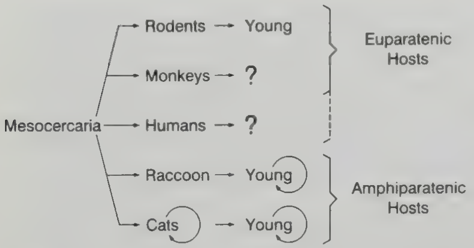


FIGURE 12. Two generalized consequences of transmammary transmission in *Alaria marcianae* as viewed in the F_1 generation of certain hosts.

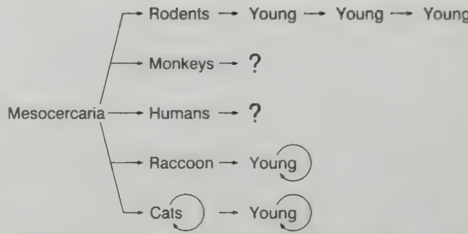


FIGURE 13. Consequences of transmammary transmission in *Alaria marcianae* as viewed beyond the F_1 generation of certain hosts.

perhaps dying only if crushed in the masticatory processes of ingestion. The spectrum of paratenesis in *Alaria marcianae* is greater than any other known helminth and involves amphibians, reptiles, birds, and mammals (including humans). This paratenic expansion in the *A. marcianae* life cycle most likely developed as a consequence of the third intermediate and definitive host telescoping into one.

The somatic exploration of mesocercariae in various amphibian, reptilian, avian, and mammalian paratenic hosts typically culminates with the localization of the larvae in fat deposits (Shoop and Corkum, 1981). In mammals, the fat deposits of preference are often subcutaneous and, hence, it became a small step before the mesocercaria discovered the mammary glands. With this came a novel form of transmission within the Trematoda. Utilizing the milk as a medium the mesocercaria explored a route to another generation; a generation that was a new set of islands, with no competitors, and few defense mechanisms. Data published initially from our lab (Shoop and Corkum, 1983a), as well as numerous experiments since, have never failed to show less than 100% infection of first litters born to infected rodent or carnivore females. We have also found the mesocercaria capable of responding to some steroid hormones and this may be the stimulus that draws them out of their hypobiotic state and to the mammary glands. Whatever the stimulus, to account for 100% infection of the nurslings it must be powerful.

In the following histological sections we get an idea of how the mesocercaria traverses the mammary glands. First, the parasite is quite capable of entering the parenchyma of the glands through direct invasion of the dense connective tissue (Fig. 9). Penetration is facilitated by 4 large unicellular penetration glands that occupy the anterior one-third of the parasite. When entering the parenchyma, the mesocercaria migrates in tracks of lipid cells rather than through the alveoli (Fig. 10). Finally, when the larva arrives in the proximity of the teat it carves out a large lactiferous duct of its own creation and comes to lie in a pool of milk (Fig. 11). At this point transmission depends upon the neonatal maelstrom (Shoop and Corkum, 1984c).

Transmammary transmission in *Alaria marcianae* has given rise to several patterns of host infection. The first pattern occurs in rodent paratenic hosts (Fig. 12). If the rodent female is lactating she will pass mesocercariae to all of her offspring where they remain mesocercariae. Therefore, both adult and neonate rodents act as paratenic hosts. However, a feline "definitive host," if lactating, will also pass mesocercariae to all of her offspring, but they then undergo that complex circuitous migration alluded to earlier, mature, and 16–18 mo later they die and are spontaneously eliminated. This type of host is called an amphiparatenic host because, depending on the physiological state of the host, it can serve as either a paratenic or definitive host.

We asked a very interesting series of questions. What happens to the mesocercariae in the second generation? Can they passage on? We found that if the second generation of rodent matured and was allowed to mate they would in turn infect members of the third generation as well—and presumably on and on it goes (Fig. 13). However, transmission in the feline amphiparatenic host ends at the second generation because the parasites initiate the ageing process in the lungs, mature in the small intestine, and die (Shoop and Corkum, 1984a).

To provide an idea of how effective this mode of transmission is, we infected a female cat with a modest inoculum (800 mesocercariae) and allowed her to bring forth a number of litters (Ta-

TABLE I. *The number of* Alaria marcianae *recovered from 8 consecutive litters born to and suckled from an experimentally infected cat (taken from Shoop and Corkum, 1987).*

Kitten no.	Number of *A. marcianae* recovered from litters born:							
	28 Jul 1982	15 Oct 1983	9 Feb 1984	27 May 1984	13 Sep 1984	16 Jan 1985*	20 Apr 1985*	18 Jun 1985
1	45	25	8	1	0	—	—	0
2	51	30	11	2	0	—	—	0
3	37	36	12	1	0	—	—	0
4	43	40	7	1	1	—	—	0
5	35	25	9	1	—	—	—	0
Totals	211	156	47	6	1	—	—	0
Mean	42	31	9	1	0.25	—	—	0

* Litters destroyed by female within 2 days of birth.

ble I). She infected every member of the first 4 litters (a total of 20 offspring) and was slowly, over the course of the parturitions, exhausted of the infection. However, even after 3 yr she had mesocercariae remaining in her tissues (Shoop and Corkum, 1987).

This form of transmission is not an aberrancy within the *Alaria marcianae* life cycle. In fact, there is some evidence that suggests it is the mechanism by which this organism is maintained in nature. Pence and Windberg (1984) have reported that rank abundances of adult *Alaria marcianae* in wild canids show pups with greater infection than juveniles, and juveniles with greater infection than adults. This age-dependent overdispersion in which young hosts harbor the most parasites is exactly the opposite population structure one should expect if transmission was dependent upon carnivory.

Although we initially coined the term amphiparatenic host only for hosts of *Alaria marcianae,* it quickly became clear that the phenomenon we were attempting to describe was not unique to trematodes. It also characterized the *Ancylostoma caninum, Neoascaris vitulorum, Strongyloides* spp., *Toxocara canis,* and *Uncinaria lucasi* life cycles, to name a few. This stimulated a renewed effort to capture the essence of the amphiparatenic strategy and again we used *Alaria marcianae* as the model (Shoop and Corkum, 1987). Included in the concept were (i) larvae that undergo a somatic migration and remain in an undifferentiated and hypobiotic state for extended periods, (ii) the dependence upon vertical transmission as a major mechanism in the maintenance of the species, (iii) pregnant or lactating females which serve as both reservoirs and vectors of infection, (iv) an overdispersed parasite population structure based on host age in which the neonates have greater numbers of adult worms than do juveniles or adults, and (v) an overdispersed parasite population structure based on host sex that sometimes appears to indicate a preference for the male. The presence of more worms in the younger age classes and in males is illusory. In actuality, the female usually harbors a greater infection because she is a reservoir for worms in her parenteral tissues. However, this fact is cryptic because those worms in the female tissues remain hypobiotic larvae and not the more readily apparent and easily recovered adults.

It is clear that with *Alaria marcianae* the larval parasites are capable of exercising options while in amphiparatenic hosts. If in a host that maternal transfer is not possible, i.e., males and offspring, then the worms mature and insure fitness. However, in a pregnant or lactating female they forego the ability for immediate maturation and opt for transmission to a new generation of hosts. The maintenance of the larval state on the part of the parasite is an adaptation for transmission and is *not* the consequence of resistance on the part of the host. This is true for all species that utilize the amphiparatenic strategy. As I argued elsewhere, it is hard to believe that a strategy resulting in the almost 100% infection of dogs on this planet with *Toxocara canis* is a benefit to the host and not to the parasite. The same ubiquity in mammals would be seen with *Alaria marcianae* were it not for the historical "baggage" of the mollusc and other required hosts in its life cycle.

To those species that practice the amphiparatenic strategy, the intimate contact required for vertical transmission represents one of the few cases of contagion in the helminthiases, i.e., direct transfer of an infective agent from one member of a host species to another with no free-living stage or vector intercalated. For *Alaria marcianae* and *Pharyngostomoides* spp., a case

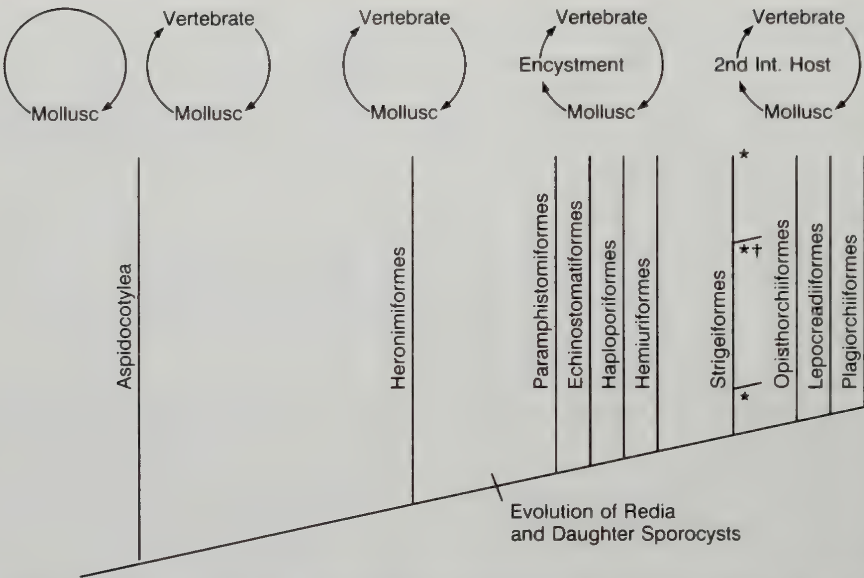


FIGURE 14. The major life cycle and transmission patterns superimposed on the phylogeny of the Trematoda (the latter redrawn from Brooks et al., 1985b). The * represents the site on this phylogeny where 4-host life cycles occur; † represents where transmammary transmission occurs; and ★ represents where a derived 2-host pattern occurs.

could also be made that milk-borne transmission is a subtle form of inoculation. In addition to "sucking," an offspring also "stimulates" the mammary gland. This sets into motion higher-order neural and endocrinological mechanisms culminating in the release of oxytocin, which causes contraction of smooth muscle and milk let-down. Therefore, it is not too farfetched to think that the milk, along with the mesocercaria, is actually inoculated into the mouth of the offspring. This would be novel, for nowhere else in the Trematoda is inoculative transmission found.

Finally, it can be said that maternal transmission in *Alaria marcianae* represents the ultimate expression of paratenesis. Not only is the mesocercaria woven into the food chain of most vertebrate classes, but it has now found a mechanism to enter the only food source common to every species of mammal. As a consequence of paratenesis, the shuttling between hosts defies categorization of the *A. marcianae* life cycle as a 3-, 4-, or 5-host life cycle. Theoretically, the number of hosts is infinite. I have collected mesocercariae from hundreds of amphibian, reptilian, avian, and mammalian paratenic hosts in the swamps of Louisiana. Most of them were probably produced the year before, and a few the year before that, but I have often wondered if one of these hypobiotic larvae could be 10,000 yr old. It is perhaps too bad that we cannot carbon-date them for they may have a few surprises for us!

Now the major life cycle patterns can be viewed as they fit the most current phylogeny (Fig. 14). We must resist the temptation to create hypothetical scenarios of life cycle evolution and then, after the fact, attempt to find taxa that fit the various steps we designed. Instead, we must allow the phylogeny of the organisms to dictate the evolution of life cycle patterns to us.

To recapitulate, our current phylogenetic data, based primarily on morphological criteria, tell us that to an original 1-host life cycle involving a mollusc a vertebrate host was added. The evolution of nonmollusc-feeding vertebrates may have doomed this early 2-host lineage to obscurity (such as in the aspidocotyleans and heronimids) except for the evolution of the redia and daughter sporocyst stages. The redia and daughter sporocyst made possible the high-risk spatial dissemination of the cercaria from the mollusc to the new group of vertebrates. Cercarial encystment and hypobiosis furthered the ability to disseminate temporally from the mollusc. En-

cystment also forecast the intercalation of the metacercarial stage and the subsequent acquisition of an additional intermediate host giving rise to a 3-host life cycle. In 2 closely related families, a 4-host life cycle has evolved and is characterized by the presence of a mesocercarial stage and the intercalation of a third intermediate host. It is from this already complex 4-host life cycle that we now see the highly derived *Alaria marcianae* cycle complete with paratenesis, hypobiosis, contagion, transmammary transmission, amphiparatenesis, and the use of host defense mechanism-facilitated transmission all rolled into one.

To sum up, this has been a brief journey through the basic life cycle and transmission patterns in the Trematoda. I have presented both because they are inextricably linked—transmission requirements dictate life cycle patterns. I hope it is evident that the only way we can truly understand these complex patterns of development and transmission is when they are placed in a proper evolutionary perspective.

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CESTODE TRANSMISSION PATTERNS

John S. Mackiewicz
Department of Biological Sciences, State University of New York at Albany,
Albany, New York 12222

ABSTRACT: The paradox of high prevalence but low probability of having an egg develop to an adult has been resolved by the evolution of 3 major and basic strategies involving transmission: evolution of life cycles interpolated into host biology; presentation of infective stages that increase probability of contact between host and parasite; and, increase in reproductive potential. The rarity of direct cycles confirms that cycles in themselves, with at least 2 hosts, are a key element of cestode success because they provide a vehicle for dispersal and transmission of infective stages. Transmission is primarily by passive stages that become incorporated through intermediate hosts or accidentally in the food chain. High host specificity results from efficient transmission pathways but may represent a fragile system for the evolution of the species. Probability of transmission is increased through diversity of intermediate hosts, making eggs more susceptible to ingestion and by behavioral manipulation of hosts by parasite stages. Spatial and temporal aspects of transmission may be increased through paratenesis. Asexual proliferation of immature stages is uncommon and is favored where there is selective predation; such proliferation may be part of a transmission strategy of colonial cestodes that require high infrapopulations in order to survive. Hyperapolysis may be part of a transmission strategy used by the Tetraphyllidea, Trypanorhyncha, and Lecanicephalidea to increase proglottid production. The dynamics of transmission for cestodes of humans and domestic animals require a different perspective than those of wild hosts. All strategies are reviewed within the framework of certain cestode morphological and ecological constraints. A total of 11 figures and 48 references complements the text.

In many ways cestodes are a paradox. On the one hand they constitute a rather common part of the endohelminth fauna of many animals. For example they make up approximately: 30% of endohelminths from North American freshwater fish, 31% of those from ducks, and 33% of those from dogs and cats. And of individual host species they constitute approximately 14% of helminths from racoons, 18% from humans, 25% from coyotes, 61% from spiny dogfish, and 100% from round stingrays. Furthermore, in some they may occur in prodigious numbers. In a single scaup duck Hair and Holmes (1975) found almost 11,000 of one species of *Hymenolepis,* over 7,000 of another, and almost 5,000 of 11 other species.

On the other hand, the probability of a single egg developing to an adult is very low. In one of the few studies that provided hard data, Jarroll (1980) found that with *Bothriocephalus rarus,* from the red spotted newt, the probability of an egg developing to an adult was 0.08%. And Ghazal and Avery (1976) found in the direct cycle of *Vampirolepis nana* in mice that the probability of infection for an individual egg was 2.9×10^{-6}. These data are consistent with the conclusion of Esch (1983) that the prevalence of cestode infection in intermediate hosts is generally low.

This paradox of high prevalence in definitive hosts, while at the same time having an intrinsic, very low probability of success in an egg developing to an adult worm, has been resolved by the evolution of complex life cycles involving interactions of many component parts—among the most vital being transmission strategies. That successful transmission is not simply something cestodes do alone but something cestodes and hosts accomplish together, with a modulating effect by environmental factors and regulatory feedback at the population level, can be readily appreciated from a reading of the papers of Kates (1965), Crofton (1971), Anderson (1976), Price (1980), Esch (1983), Kennedy (1983), and Holmes (1987). Since these papers provide ample information on various aspects of transmission, this paper will briefly touch upon what I consider to be basic transmission strategies as viewed within the perspective of the severe constraints posed by the unusual morphology and biological characteristics of cestodes.

PERSPECTIVE

With all parasitic organisms, the nature and evolution of transmission strategies—indeed all strategies—should be assessed in light of basic characteristics of the parasite. By lacking a gut the adult tapeworm, unlike nematodes and trematodes, is unable to exploit such diverse environments as the lung, kidney, or the circulatory system and is thus relegated to the intestine as its sole habitat. With this constraint of a single habitat came enormous benefits—an environment of unlimited, even superabundant food resources that, as Jennings and Calow (1975) postulated, had an automatic consequence: the

evolution of organisms with little limitation on high fecundity, an ideal condition for an r-selection strategist. Furthermore, living in the lumen of the gut does not usually trigger pronounced immune responses thus enabling the same host to be repeatedly exploited over the course of its life. Both of these characteristics of the intestine—superabundance of nutrients and reduced immune responsiveness—have had profound consequences for the evolution of transmission strategies. On the other hand, the restricted environment may make cestodes more responsive to the effects of intra- and interspecific competition for space and nutrients and thus: to the extent that infrapopulation and community structure are influenced by such effects so too would be the transmission dynamics.

By lacking a free-living stage (except for a short-lived, weakly swimming coracidium [Fig. 4] in the Pseudophyllidea and some Trypanorhyncha), eggs, and even coracidia, are basically biological nomads, undirected and passive with respect to prospective hosts. Transmission strategies with these stages, must, therefore, be basically passive in nature. Furthermore, by having the oncosphere as the only larval stage common to all tapeworms, they have been freed of evolutionary baggage, and thus, unlike trematodes, have been free to evolve various larval stages and establish diverse evolutionary lines. Since none of these other stages before the adult are free living, being in tissues, organs, or the body cavity, transmission strategies involving them must also be passive, as are recruitment strategies (Esch, 1983).

Unlike trematodes who are forever bound to molluscs for part of their life cycle, cestodes, by their very nature are freed of an evolutionary history that ties them to any specific organism thus allowing them to exploit a large variety of invertebrates and vertebrates as intermediate hosts. The trophic and ecological relationships between this large array of diverse intermediate hosts and definitive host thus assumes a prominent role in cestode transmission strategies. Density-dependent and environmental factors that help regulate the interactions of hosts through predator–prey associations can also be expected to influence cestode transmissions that function through those associations.

TRANSMISSION STRATEGIES

Successful transmission from host to host rests at the core of parasite survival. Cestodes continue to flourish, in fact thrive, through the evolution of 3 major and basic transmission strategies:

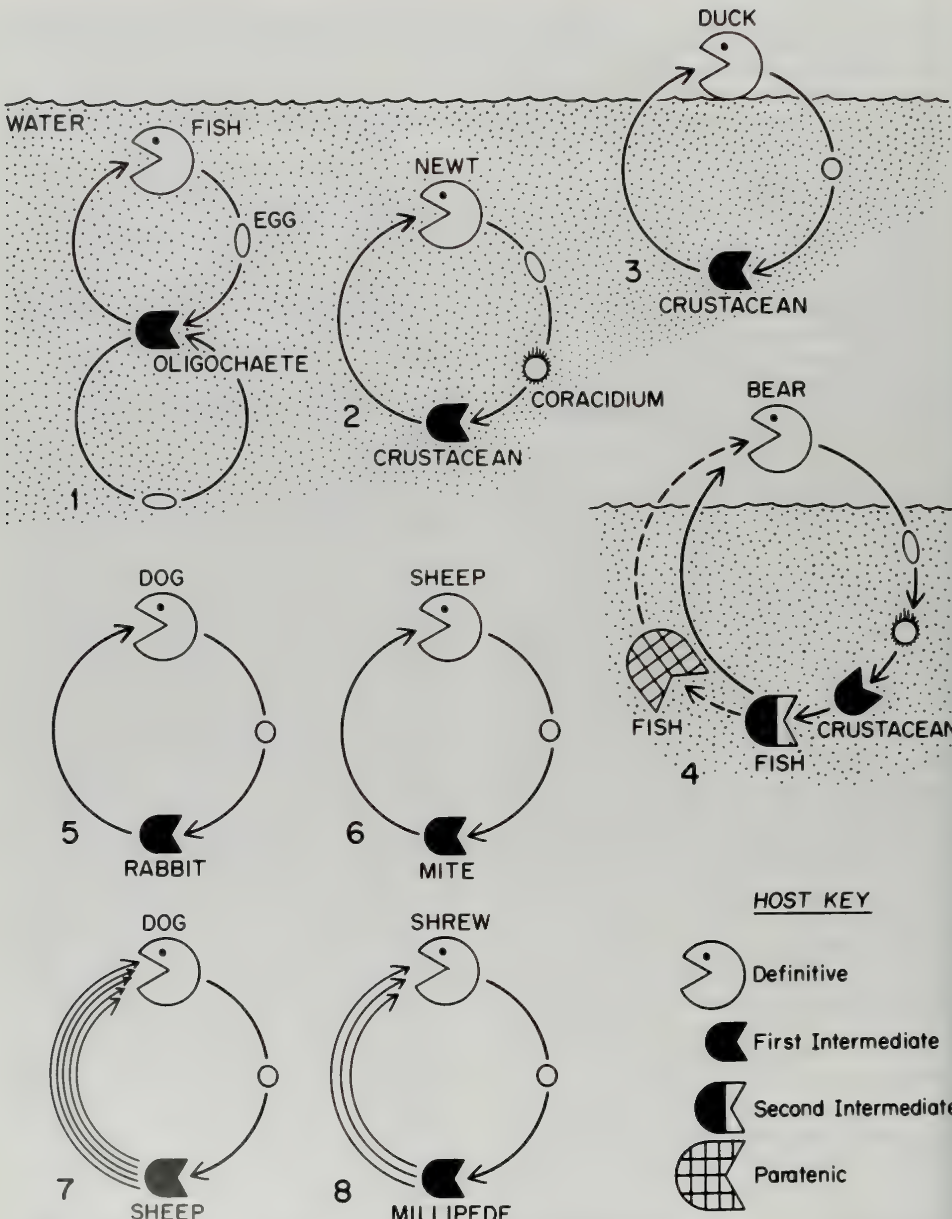
I) Evolution of life cycles interpolated into host biology.
II) Presentation of infective stages that increase the probability of contact between host and parasite.
III) Increased reproductive potential.

Strategies I and III are clearly characteristic of all cestodes while II is in part a consequence of I and III and in part operating directly through intermediate hosts.

I. EVOLUTION OF LIFE CYCLES INTERPOLATED INTO HOST BIOLOGY

Life cycles

The evolution of the life cycle with diverse stages is a hallmark of cestode biology (Figs. 1–8). These life cycles can be arranged in various ways: aquatic (Figs. 1, 2), terrestrial (Figs. 5–8), or amphibious (Figs. 3, 4). Or, as is traditionally done in textbooks by the number of hosts: 1, 2, or 3. The 3-host cycles, *Diphyllobothrium* for example (Fig. 4), appear to be more common in aquatic or amphibious situations. In terrestrial ecosystems, the single intermediate host or 2-host cycles are dominant and exemplified by such genera as *Taenia* (Fig. 5), *Moniezia* (Fig. 6), and *Hymenolepis.* All have been successful but if numbers are any guide, the direct or 1-host cycle has been the least successful since *Vampirolepis nana* in mice and *Archigetes* (Fig. 1) in aquatic oligochaetes are all we know. Both cycles are facultatively direct, with *V. nana* also capable of internal autoinfection from ruptured proglottids in the gut. The cysticercoids of *V. nana* normally develop in beetles, the intermediate host, while *Archigetes* can also mature in fish. The *Archigetes* cycle, the only one we know with an invertebrate definitive host, is actually an abnormal one because of progenesis, or sexual maturity of a larval stage, and is not the ontogenetic equivalent of *V. nana* although both produce eggs (Mackiewicz, 1981). To what extent the direct cycle contributes to the cestode population in feral, free-ranging house mice or rats is not known. It is probable that the direct cycle would be largely supplanted by the 2-host cycle because of the enhanced transmission through intermediate host dispersal and the rapidly developing immune response associated with the tissue-dwelling cysticercoid stage.



FIGURES 1–8. Life cycles. **1.** *Archigetes iowensis* (Caryophyllidea), 2-host and direct cycle. **2.** *Bothriocephalus rarus* (Pseudophyllidea). **3.** *Fimbriaria fasciolaris* (Cyclophyllidea). **4.** *Diphyllobothrium latum* (Pseudophyllidea), 3-host cycle with optional paratenic host. **5.** *Taenia pisiformis* (Cyclophyllidea). **6.** *Moniezia expansa* (Cyclophyllidea). **7.** *Echinococcus granulosus* (Cyclophyllidea), with great larval proliferation in hydatids in vertebrate intermediate host. **8.** *Pseudodiorchis prolifer* (Cyclophyllidea), with larval proliferation in staphylocyst in invertebrate intermediate host.

In addition to the serious problems posed by host mobility, there would have to be a greater prevalence of coprophagy or opportunities for egg ingestion for direct cycles to be more widespread. Certainly, host behavior of all but mud- or detritus-feeding fish would mitigate against the evolution of direct cycles with aquatic definitive hosts. Coupled with the fact that egg infectivity may be low, as reported by Lawson and Gemmell (1983) in *Taenia,* thus necessitating large doses, the behavioral characteristics of the vertebrate host, and a probable adverse immune response from parenteral or tissue-dwelling larval stages offer attractive clues to why direct cestode life cycles have not been selected for. It would seem that evolution may not be toward more simple or even direct cycles because the probability of transmission success is lower, paradoxically, than in the more "complex" 2- and 3-host cycles. The extreme rarity of cestodes with direct infection routes helps confirm that the cycle pattern in itself, involving at least one other host, is a key element of the strategy of cestode success, largely because it provides a vehicle for transmission and dispersal of infective stages.

The incorporation of various life stages between egg and adult has had far-reaching consequences for successful transmission. These additional stages, metacestode or cercoid, depending on whether you follow the terminology of Freeman (1973) or Jarecka (1975)—I will call them larval stages for convenience—greatly expanded the host's capacity for dispersal. Without larval stages in another organism, dispersal in space would be limited to the route taken by the host as it deposits parasite eggs in the environment, and dispersal and dissemination, or the release of eggs from a proglottid, essentially become the same process. It is well known that some expelled proglottids have limited capacity for locomotion away from the fecal mass or host. As reviewed by Lawson and Gemmell (1983), segments of *Echinococcus granulosus* migrated "considerable distances" and even climbed vertically on plants; those of *T. hydatigena* migrated 90 cm horizontally away from feces. I have observed a proglottid of *Dipylidium caninum* crawling on a couch cushion not far from our sleeping cat. The adaptive significance of motile proglottids would appear to be to disperse eggs to noncoprophagous intermediate hosts thus increasing host diversity. Just how widespread is such proglottid behavior is not known. However, the evolution of cycles with larval stages has resulted in the separation of dispersal from the limited process of dissemination, thus allowing for a separate strategy of dispersal in time and space. Without this potential for expanded modes of dispersal, diverse strategies for transmission would be limited.

Compared to the egg stage, which generally survives for less than year in many cestodes, larval stages have a greater longevity. Whereas the eggs of *Echinococcus* may be able to survive for up to 4 yr, the larval hydatid stage may live for over 10 yr in a variety of vertebrate hosts including humans. In 3-host cycles such as *Diphyllobothrium* (Fig. 4) the combined longevity of the procercoid and plerocercoid stages may be several years as compared to the less than a year for the eggs alone. The net benefit of this longevity is that the window of vulnerability or exposure for transmission to the definitive host is increased from a peephole to a picture window. Furthermore, infective cestode stages continue to circulate in the environment even when hosts are not available. Until a fox or coyote catches an infected rabbit, the cysticerci of *Taenia pisiformis* (Fig. 5) remain waiting in the body cavity undergoing no further development. In *Diphyllobothrium* the plerocercoid may move up the trophic ladder through a series of paratenic hosts, thus further enlarging the time window for dispersal. In aquatic cycles where there is apt to be a strong seasonal distribution of infected zooplankton, that restricted availability to hosts is stretched out beyond the seasons because of other stages in longer-lived fish and paratenic hosts. In order to exploit the seasonal or periodic availability of aquatic intermediate hosts, the Pseudophyllidea have developed short duration and synchronous egg release as part of their transmission strategy. Large numbers of eggs are liberated in a short time just when suitable zooplankton become abundant. Where season may not be a strong factor, as in terrestrial cycles with predator–prey links, the strategy is more apt to involve long duration and nonperiodic egg release as is evident in the Cyclophyllidea. Here proglottids may be released continuously for periods that may stretch for years or over the life of the host. The ecological significance of short duration or semelparity and long duration or interoparity egg release patterns in cestodes has been discussed in detail by Kennedy (1983). Whichever egg release strategy is used, short duration (that may be synchronous) or long duration, the net effect is to produce passive larval stages that allow cestodes to survive longer and

thus adapt a grand transmission strategy of sit and wait. Where there is such a strategy having long-lived transmission stages, May (1983) has concluded that the threshold host densities for maintenance of parasite populations can be small. Since predator–prey links are present in so many cestode cycles there is little wonder that a waiting transmission strategy has evolved.

Dispersal in space is a natural consequence of intermediate hosts carrying larval stages beyond the initial source of infection. Such dispersal is especially significant and highly successful in circulating cestodes in a predator–prey system, such as the canid–rabbit cycle of *Taenia pisiformis* which occurs over a broad geographical area. In the 3-host amphibious cycle of *Diphyllobothrium* there may be spatial dispersal that results in a partitioning of host species, thus allowing for several species with rather similar cycles to succeed in the same ecosystem. Halvorsen and Wissler (1973) found that 3 species of *Diphyllobothrium* occurred in different hosts depending on the extent a paratenic host was used in the cycle. *Diphyllobothrium latum* used successive paratenic hosts, finally reaching a large fish that was then harvested by humans, the definitive host. On the other hand, *D. dendriticum* and *D. ditremum* incorporated paratenic hosts to a limited extent with the result that plerocercoids occurred in smaller fish that were then suitable prey for piscivorous birds such as gulls and loons. Thus, by using paratenic hosts as a dispersal strategy prey size becomes a strong influence on patterns of host specificity. Paratenic hosts are particularly important in traversing adjacent links in community tropic webs in marine epipelagic environments and may help account for the disproportionate numbers of cestodes in elasmobranch fishes. For example in about 150 species of elasmobranch fishes in Atlantic coastal waters Campbell (1983) found more than 400 species of adult cestodes representing 5 orders. Paratenesis, or the incorporation of paratenic hosts as part of a transmission strategy, is also widespread in the nematodes (Anderson, 1988).

Life cycles and host biology

After the evolution of life cycles their interpolation into the normal biology of the host becomes the dominant strategy for successful transmission. This interpolation has largely been achieved by utilizing the food and food chain of hosts. As Chandler (1955) long ago observed, in order for a parasite to live habitually in a host, 2 conditions must be satisfied: (1) there must be a dependable means of transfer from individual to individual; and, (2) the parasite must have the ability to thrive once it gets in the host. By parasitizing the food chain, cestodes have the potential of becoming as intimate a part of the host's biology as its breeding habits or behavior. To be sure, such a transmission strategy is not without risks, because the process is wholly passive with the parasite having little or no control over choice of host.

The benefits of parasitizing the food chain are far more advantageous when compared to the risks of survival in a direct cycle. At each trophic level there are a great variety of primary, secondary, or tertiary consumers that potentially can serve as intermediate hosts or vectors of eggs. A partial list of invertebrates implicated in cestode cycles includes: various species of oribatid, tyroglyphid, and other mites; numerous beetles, grasshoppers, ants, psocids, collembolans, mecopterans, many types of flies, mallophagans, caterpillars, earwigs; crustaceans such as copepods, amphipods, ostracods; millipedes; leeches, earthworms, aquatic oligochaetes; and molluscs (marine, freshwater, and terrestrial). For an extensive discussion of the invertebrates associated with *Taenia* life cycles see Lawson and Gemmell (1983). While not strictly part of the food chain of vertebrates, mites, psocids and other very small arthropods are accidentally eaten on forage crops thus allowing herbivores, that otherwise would have to be part of a direct cycle, to become definitive hosts (Fig. 6). In this way anoplocephalids have been able to evolve in a wide range of ruminants and other herbivores.

One must not forget the vertebrates too can serve in the pool of intermediate hosts as evident in the predator–prey cycles of so many of the Cyclophyllidea (Figs. 5, 7) and Pseudophyllidea (Fig. 4). By being at the top of the food chain, a predator becomes a kind of parasite sink that allows for unusual cestode diversity as a result of various transmission pathways involving different prey and types of larvae. In parts of the U.S.S.R., a single predator host species, the dog, serves as definitive host for 7 species of *Taenia*, 4 of *Multiceps*, and 1 each of *Echinococcus*, *Alveococcus*, and *Hydatigera* in cycles with 5 larval types and 14 different vertebrate hosts (Abuladze, 1964). Thus, the nature of the food web and transmission pathways can strongly influence how cestode and host diversity is expressed.

Host specificity

The relationship of host specificity to patterns of transmission is an interesting one but is difficult to assess because of the absence of experimental studies. From a theoretical point of view it would seem that any factors that limit the kinds of hosts a cestode can reproduce in would limit the success of a species and thus have negative selection pressure. High host specificity, often associated with cestodes, would then seem to mitigate against survival. However, if one views host specificity as evidence of a proven cycle where transmission patterns have been so consistent and efficient as to result in physiological specificity, then barring any unforeseen changes in the cycle, the species should continue to be successful. In this sense host specificity minimizes a certain amount of risk because there is a template for success, and thus it may actually favor the survival of the species. What it does not favor, however, is the evolution of the species because it has lost much of its capacity to exploit new hosts. Even if transmission patterns are efficient, cestodes with high host specificity represent a fragile system that can tolerate little perturbation such as major changes in host diet or worse still, loss of host from the former range of the parasite. Should the host become extinct, so too would its highly host-specific cestodes.

Aside from theoretical considerations, there are more practical ones dealing with host specificity and successful transmission. There seems little doubt that it must be analyzed on a species-by-species basis and that often it can not be studied solely from literature records. We know for example that mature *Taeniarhynchus saginatus* have been found only in humans and *Marsipometra* only in *Polydon,* the spoonbill catfish. At the other extreme is *Fimbriaria fasciolaris,* a cosmopolitan cestode of waterfowl and other birds, that has been recorded from 63 birds in 8 different orders. And then there is *Vampirolepis nana* the common dwarf tapeworm of house mice, rats, and occasionally humans. In this last instance literature records would seem to indicate perhaps moderate host specificity. However, the data from natural infections are often quite different than those from experimental ones. Schiller (1959) experimentally infected 41 different mammals from 6 orders and found that the cestode matured in 12 different hosts; in the case of the woodchuck and grey squirrel development was better than in the normal, mouse host. These data would suggest that there appears to be built into some life cycles a much wider range of hosts than is normally expressed—that is, unless transmission pathways are altered through environmental stress or changes in host behavior. This strategy of having potential or unrealized host capabilities gives some cestode cycles intrinsic resiliency. The greater the number of possible transmission pathways the greater the resiliency. Schiller concluded that the apparent host specificity, as judged by literature records, was not physiologically based but more related to feeding behavior: woodchucks and grey squirrels were not infected because they simply did not share the same food items as rats and mice. His study once again emphasized the central role that diet and feeding habits play in the successful transmission of cestodes and how, by extension, modification of that diet may lead to a greater host spectrum.

Because there can be less host specificity at the invertebrate host level, some cestodes develop in and are transmitted by a wide variety of invertebrates thus greatly extending their range. For example, Jourdane (1975) found that there were 10 different intermediate hosts for *Hymenolepis furcata,* a parasite of a widely distributed European shrew: in Poland there were 2 genera and species of beetles; in Czechoslovakia, 5 genera and 6 species of beetles; and in the Pyrenees of France, beetles are replaced by a mecopteran and a millipede. Such an example dramatically illustrates how through different transmission hosts it is possible for exotic cestodes to establish themselves in new regions, viz. *Khawia sinensis* from Asia to eastern Europe and *Bothriocephalus acheilognathi* from Japan to Europe and the United States.

II. PRESENTATION OF INFECTIVE STAGES THAT INCREASE THE PROBABILITY OF CONTACT BETWEEN HOST AND PARASITE

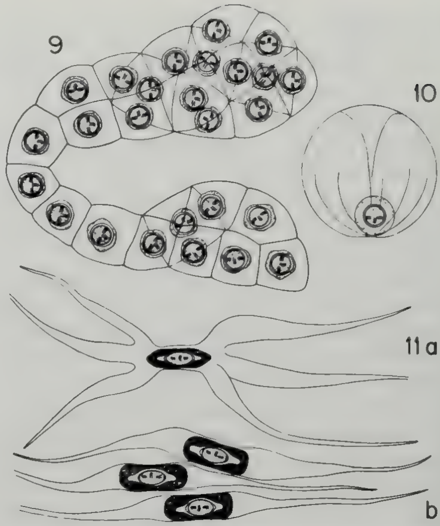
Intermediate host diversity

There are a number of factors or conditions that will increase the probability that transmission will be enhanced. One of these is an increased number and diversity of intermediate host species. We have already seen that *Fimbriaria fasciolaris* from waterfowl is an immensely successful tapeworm having a cosmopolitan distribution and reported from 8 bird orders and 63 hosts, of which 53 are ducks. A closer look at its 2-host cycle reveals that the intermediate hosts include 19 species of copepods, ostracods,

and amphipods. In contrast, another cosmopolitan species from waterfowl, *Cloacotaenia megalops,* is reported from only 3 orders and 43 hosts of which 41 are ducks. In this case only 1 species of ostracod appears to be the intermediate host. These data are consistent with Avery's (1969) data that demonstrated the infection efficiency of cestode larvae to ducks was from 3 to 20 times greater when a cestode had both ostracod and copepod intermediate hosts, than when it had only ostracods. However, that such a direct relationship between the number of intermediate hosts and diversity of hosts is only a trend is borne out by *Myxolepis collaris,* with 4 transmission hosts that include 12 species of copepods, ostracods, and amphipods as well as 2 species of lymnaeid snails that harbor cysticercoids after feeding on infected ostracods. Surprisingly, *Myxolepis* is reported from 4 orders and 26 hosts, of which 23 are ducks, fewer hosts than either *Fimbriaria* or *Cloacotaenia.* Clearly successful transmission to a wide diversity of hosts entails factors other than just having a large number of different intermediate hosts.

Egg adaptations

Some of these factors are intrinsic to the cestode, others concern the effect of the cestode on the host. Jarecka (1961) discovered that some cestode eggs in aquatic ecosystems have remarkable adaptations (Figs. 9–11) that no doubt increase the probability that they will be ingested by a wide variety of intermediate hosts. For example: the large, heavy packets of *Hymenolepis abortiva* (Fig. 9) sink, are too big for zooplankton, but are attractive food items for oligochaetes and amphipods; those of *Proteocephalus percae* (Fig. 10) float, are not joined, and because of their large size are eaten by large copepods; and, the filiform eggs of *Diorchis* spp. (Fig. 11) mimic diatoms and are eaten by ostracods as they graze aquatic plants contaminated with these eggs. The similarity of the heavy egg packets of a number of species, such as *Fimbriaria fasciolaris* to filamentous algae probably increases the probability of ingestion by benthic amphipods (Podesta and Holmes, 1970). Whether such adaptations exist in tapeworms with terrestrial hosts can only be guessed at, however, Berntzen and Voge (1962) found 2 morphologically distinct types of eggs from individual proglottids of *V. nana.* One type hatched only in extracts from *Dermestes* beetles while the other hatched in the same extract and in a solution of trypsin, urea,



FIGURES 9–11. Eggs of cestodes from waterfowl illustrating adaptations for selective ingestion. **9.** *Hymenolepis abortiva* (Cyclophyllidea), large egg packet. **10.** *Proteocephalus percae* (Proteocephalidea), large floating egg. **11a.** *Diorchis* sp., and **11b.** *Diorchis nyrocae* (Cyclophyllidea) that mimic diatoms. (All Figures after Jarecka, 1961.)

and bile salts—the latter phenomenon an apparent adaptation for the direct cycle. Recently Pappas and Leiby (1986) also found 2 types of eggs of 4 "strains" of *H. diminuta* that were significantly different in size and separated out in 2 regions on a sucrose gradient. While experimental feeding to *Tenebrio* beetles showed no differences in infectivity, one wonders if such size differences might allow for selection of different insect hosts under field conditions. Where eggs in terrestrial cycles are eaten as food items, as in the case of anoplocephalids (Fig. 6), rather than as contaminants of food (Fig. 5), it may be that subtle mechanisms such as chemical attractants increase the probability of eggs being eaten by oribatid mites. Or, is high fecundity sufficient to insure infection of intermediate hosts? It is difficult for me to believe that all cestode eggs deposited in terrestrial cycles are eaten accidentally or incidentally with the remains of a proglottid, much as we would swallow seeds while eating watermelon. On the other hand, so prodigious is egg production and so abundant are intermediate hosts that, with anoplocephalids at least, chance encounters between egg and invertebrate host may well be the primary transmission strategy, uncomplicated by any special egg adaptations. Aside

from a few studies such as that of Jarecka (1961), we know little of the adaptive significance of egg form and of the dynamics of egg predation—yet it is with the egg that transmission starts and where mortality can be very high.

Behavior manipulation

Host behavior may also influence transmission success. Since cestodes can not be opportunistic because of their passive stages, one strategy left to them is to optimize transmission by accentuating infected intermediate hosts through behavior manipulation. The probability that a predator will detect a prey increases once the behavior or appearance of the prey deviates appreciably from the norm. Such deviations have been caused by parasites including larval cestodes and may result in general enhancement of transmission through selective predation, a subject reviewed by Holmes and Bethel (1972). Such behavioral manipulation by a parasite is termed "host suicide" by Trail (1980) and figures prominently in her analysis of the evolution of complex parasite life cycles.

There is great variation on the effects of larvae and much depends on the number of larvae and nutritional status and age of the host. Sticklebacks infected with large *Schistocephalus* plerocercoids, often large enough to deform the host, will swim closer to the surface and prefer shallower water, thus becoming easier prey for their bird predators (Lester, 1971). *Ligula* in minnows may cause a similar behavioral alteration (Dence, 1958) as well as a hormonal imbalance in the host. In terrestrial cycles the coenuri of *Multiceps* can lodge in the brain or spinal cord of sheep or other ruminants, where they cause an array of symptoms from staggering to paralysis, making such hosts easy targets for predators (Leiby and Dyer, 1971). Heavy infections of coenuri in snowshoe rabbits hinder movements and prevent their change in fur color, presumably to protective white coloration, ". . . making these animals easy prey to canids," according to Leiby and Dyer (1971, p. 202). While data may be conflicting, Mech (1966) has concluded that moose heavily infected with hydatids are more vulnerable to wolves. Pulmonary hydatidosis may be a special factor, in concert with others, that may lead to wolf predation on moose according to McNeill and Rau (1987). And in field mice with long-standing infections of *Echinococcus multilocularis* the abdomen becomes greatly swollen thus impeding movement (Leiby and Dyer, 1971). Mice infected with *Spirometra* plerocercoids become conspicuously obese, and would probably have more difficulty escaping predators—cats—than their sleek, trim, and speedier siblings.

Although the *Spirometra* example is from experimental infections (Mueller, 1974), it nonetheless dramatically illustrates how parasites (viz. cestode larvae) can effectively alter host appearance and behavior, thus directing selection to their advantage. A common feature of the other examples is the predator–prey relationship, that is, one organism (predator) selecting another (prey). Selective foraging by the definitive host is thus a central feature of this transmission strategy, one that may be more widespread than normally believed. Since natural selection would tend to favor such a strategy there is no reason to doubt that behavioral manipulation, to use Trail's term (Trail, 1980), also occurs in invertebrate intermediate hosts that are targets of selective foraging. Over all, by biasing predation for infected prey we have a sort of optimal foraging strategy that favors parasite selection through a parasite transmission strategy.

As impressive as all these adaptations or strategies might be, none would succeed if it was not for the grand strategy of increased reproductive potential.

III. INCREASED REPRODUCTIVE POTENTIAL

Proglottids

Whether it is *Protogynella* from shrews, less than a mm long and one of the smallest tapeworms known, or the behemoth from whales, *Tetragonoporus,* up to 30 m long with 45,000 proglottids, proglottid formation provides cestodes with a conspicuous means of increasing fecundity. It is axiomatic that the greater risk of survival or completing a cycle, the greater the reproductive potential. Where the risks during transmission are very high as in the vastness of the ocean, proglottid formation or development may be altered to further increase fecundity. In addition to the double sets of genital organs per proglottid of *Diplogonoporus* from whales there is additional asexual reproduction by transverse subdivision of each primary proglottid that more than doubles the number of proglottids and increases egg production as much as 10 times according to Rausch (1964). He speculated that production of fewer primary proglottids may be

a mechanism that enhances longer survival in the long-lived whale hosts. Such secondary proglottid formation appears to be unique to *Diplogonoporus* where it contributes to fitness for survival under conditions of great isolation and where transmission problems are extreme.

Another proglottid associated phenomenon related to transmission in a marine ecosystem may be hyperapolysis, the detachment of proglottids before they are gravid with subsequent maturation free of the strobila. It is found extensively in the Tetraphyllidea, Lecanicephalidea, and Trypanorhyncha, the major groups of cestodes of elasmobranchs, representing approximately 15% of known cestodes. According to Campbell (1983, p. 505), "The small size and hyperapolysis of so many species of elasmobranch cestodes may be the result of selection to minimize the effects of inter- and intraspecific competition." An alternative interpretation of the adaptive significance of hyperapolysis may be that it is part of a transmission strategy that allows for a greater investment of energy into proglottid production rather than into the growth and maintenance of older ones. Maximizing proglottid production would be one method of increasing reproductive potential for these small cestodes that as far as we know do not have proliferative larvae.

Proliferative larvae

Perhaps the next best way of increasing reproductive potential after proglottid production would be to have the larval stages also reproduce, as in the Trematoda. Consider the following. The number of eggs produced per day per cestode obviously varies a great deal from up to 720,000 for *Taeniarhynchus saginatus,* 45,000 for *Hymenolepis microstoma,* to 800 for *Echinococcus.* Considering that *T. saginatus* may produce about 5 million eggs per week, may be up to 18 m long, and live for 10–30 yr, the reproductive potential is staggering. However, as impressive as these figures may be they pale when compared to *Echinococcus* (Fig. 7), less than a puny 10 mm long and with only 3 proglottids. According to Smyth (1964), the reproductive potential for this species, including eggs and protoscoleces in a hydatid of 5 cc volume, is about 25,000,000/day or 5 times what *T. saginatus* produces in a week. With such an increase in reproductive potential one may wonder why some cestodes have adopted a transmission strategy involving asexual proliferation in larval stages and others, sometimes quite similar ecologically, have not. Surely more must be better—or is it?

If we assume that there are about 4,000 described species of cestodes, 3,806 are listed by Schmidt (1986), less than 1% have proliferative or asexually reproducing larvae. This is especially striking when compared to the trematodes where probably 100% of species have asexual generations. The majority of proliferative larvae are in the Cyclophyllidea and include such common and familiar species as *Echinococcus granulosus, Taenia crassiceps, Taenia multiceps,* and *Taenia serialis,* all typically in various canids and with a predator–prey type cycle. Less familiar species include *Polycercus lumbrici* (shorebird–earthworm cycle), *Staplylepis cantaniana* (pheasant, quail–dung beetle cycle), and *Pseudodiorchis prolifer* (Fig. 8) and *Staphylocystis pistillum* from shrews and with the pill millipede (Diplopoda) as intermediate host. According to Moore (1981), the only common features that taeniids with proliferative larvae have appears to be that the mature stage is relatively small, short-lived, and produces resistant eggs at a high rate. Egg and longevity characteristics of the other species are not known, however, all are small worms with *P. prolifer* being barely a mm long. We have already seen that hydatids and coenuri may make prey more vulnerable to predation and that individual predators could thus be infected with unusually high numbers of small cestodes, over 10,000 in the case of *P. prolifer* according to Kisielewska (1961). Infections of this magnitude are easily attained because the same author found a prevalence of 10% (n = 1,047) with an intensity of several thousand larvae in a single *Glomeris* (pill millipede), the intermediate host (Fig. 8). By magnifying the overdispersion of parasites in a single prey, predators in low density would have a greater chance of sustaining high enough infections for the cestode to complete its cycle.

One can, however, postulate some other benefit of this proliferative larval strategy. Since all the scoleces of a hydatid, coenuri, polycercus, or staphylocyst are of the same genotype, one wonders if it would be proper to view *Echinococcus,* and probably other species with proliferative larvae, as a social or colonial cestode that may not be able to maintain itself if they occur only in small infrapopulations. If this is the case, then perhaps the proliferative propagation of larvae is part of a transmission strategy, evolved in small

colonial cestodes, that need large infrapopulations in the host to survive, population levels that could not be attained if prey contained only a few cestode larvae. On the other hand, a large (over 2 m) robust nonproliferative species, such as *Taenia pisiformis*, can produce sufficient eggs to maintain the species so that the preferred strategy is simply to spread out egg production (iteroparity) and have a less overdispersed (aggregated) distribution of the cysticerci in rabbits. With greater dispersion of larva there would be a greater probability of a predator catching an infected prey.

In systems with predator–prey links in parasite transmission, Keymer and Anderson (1979) found that predator response in the *Tribolium–H. diminuta* system, acts as a density-dependent constraint on parasite population growth. Even when egg density was very high, the number of cysticercoids per beetle was influenced by the feeding behavior of beetles as well as survival and infectivity of eggs. Where proliferative larvae are involved, however, predator responses probaby would have little effect on parasite population levels because larval reproduction is independent of predator influences. As a result, parasite populations in a definitive host can become very large—up to 6,000 or more in the case of *E. granulosus*—thus, the apparent lack of population regulation through host behavior also becomes part of the transmission strategy. With many if not most other nonproliferative cestodes, very high parasite loads might prove harmful to the host or result in a crowding effect on cestodes. Selection in this case has thus favored some population regulation through the complex interactions in trophic webs. In effect it would seem that the transmission strategy that is good for *Echinococcus* (Fig. 7), and possibly other cestodes with proliferative larvae, is too much of a good thing for most other cestodes. In the case of *T. pisiformis* (Fig. 5), more (in the form of proliferative larvae) is not better. In place of having more than one part of the cycle make up for the risks of survival, it is the interaction of all parts of the cycle that make it work. Such nonproliferative systems are probably more stable because each part of the system can be fine-tuned to respond to fluctuating conditions of the ecosystem and host. Cycles dependent on a transmission pathway involving proliferative larvae, on the other hand, may not respond as quickly and thus they have evolved in limited predator–prey situations.

COMMENTS

When domestic animals and humans as opposed to wild hosts are involved in cestode infections, transmission strategies must be viewed somewhat differently. Because it is possible to have some control over density-dependent and density-independent factors in the management practices of domestic animals, the dynamics of transmission can be directed to the benefit of host and of animal owner. Socio-economic factors such as farming practices, feeding behavior, legislation, and education also can be important factors related to transmission as in the epidemiology of taeniid cestodes in the natural environment (Gemmell, 1987). In some instances, strong cultural factors have played a major role in transmission such as with hydatid disease among the Basque sheep herders of California (Araujo et al., 1975). This example as well as that concerning neurocysticercosis in parts of Latin America help to emphasize the importance of host behavior and food habits in cestode transmissions in human populations.

Perhaps the greatest versatility and complexity of cestode transmission patterns is exemplified by *Mesocestoides corti*, which has used existing transmission strategies, elaborated on them, and even introduced new ones. Although the first intermediate host is not known for *M. corti*, its cycle is probably very much like that of *M. lineatus*. The larval stages, tetrathyridia, occur in an unusual array of second intermediate hosts that include mammals, birds, reptiles, and even amphibians. Some of these apparently also can be paratenic hosts. In these vertebrate intermediate hosts there is asexual proliferation that occurs in various organs as well as in the coelom. When the predator definitive host ingests the tetrathyridia there is additional proliferation as Eckert et al. (1969) found when they infected a dog with 1,000 tetrathyridia and harvested over 40,000 adults. But the story does not stop here. Experiments by Conn and Etges (1983) have shown that tetrathyridia can also be transmitted from mother to suckling mice via the transmammary route. This pattern of transmission, more common in nematodes and trematodes (Miller, 1981; Shoop, 1988), is apparently not rare since up to 62% of young nursing mice were infected this way. Apparently the tetrathyridia are easily carried by the circulatory system to various parts of the body, including the mammary glands. It did not appear, however, that they were able to

cross the placental barrier and infect mice *in utero.* Given the capacity of some cestode larvae, such as *Proteocephalus* plerocercoids, to burrow through tissue, it is only a matter of time that some cestode, with a cycle very much like that of this *Mesocestoides,* will achieve that ultimate goal in transmission strategies—congenital transmission from mother to fetus. Perhaps it is already in a cycle waiting to be discovered. As pointed out elsewhere in this symposium (Anderson, 1988), nematodes have already achieved that goal.

CONCLUSION

Many interrelated factors from host food habits and behavior, population dynamics of host and parasite, and reproductive potential of parasites, to the effects of environmental factors on host and parasite, interact in symphonic fashion to insure that a cestode species will continue to exist. Strategies used to insure that existence are highly variable because of the dynamic nature of the evolutionary process and the inherent variability of biological associations. It is their fate that cestodes are prisoners of the intestine, but by evolving life cycles that have become interpolated into the biology of hosts, by enhancing the probability that cycles will be completed, and by greatly increasing their reproductive potential, they have been able to devote a large share of their energy and evolutionary cunning to solving one of the most vital and basic problems all parasites face—transmission from host to host—and in this cestodes have done very well indeed.

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LIFE HISTORY AND POPULATION BIOLOGY OF ADULT *ACANTHOCEPHALUS LUCII* (ACANTHOCEPHALA: ECHINORHYNCHIDAE)

John Brattey*

Department of Zoology, University of Liverpool, Brownlow Street, Liverpool, England L69 3BX

ABSTRACT: The life history and population biology of adult *A. lucii* in perch, *Perca fluviatilis* L., from the Forth and Clyde canal, Scotland, was investigated during May 1979–September 1981. There was an annual cycle in the size of the parasite population; prevalence and abundance (±SE) were highest during late spring and summer (70–90% and 14 ± 4.3 to 16 ± 5.6 worms/fish, respectively) but declined during late autumn and reached a minimum during winter (50–60% and 2.1 ± 0.9 to 3.2 ± 0.6 worms/fish). Parasite maturation was associated with higher water temperatures during spring and summer and most shelled acanthors were probably produced during summer and fall. There was only 1 generation of *A. lucii* per year, although generations tended to overlap and individuals within each generation did not develop synchronously. The sex ratio of adults was initially near unity but favoured females in the later stages of the infection. The distribution of *A. lucii* among perch was highly aggregated and stomach content analysis suggested that this was partly due to heterogeneity in perch feeding behaviour. The negative binomial and Poisson lognormal models fitted the data on worm distribution. Seasonal changes in the degree of parasite aggregation were detected, but no conclusive evidence of density-dependent controls on parasite population growth was obtained.

Acanthocephalus lucii (Müller) is a common and widely distributed acanthocephalan parasite of freshwater fishes in the western Palaearctic region (Petrochenko, 1958; Yamaguti, 1963; Kennedy, 1974). Although the adults infect at least 36 species of fish, the principal definitive host is the European perch, *Perca fluviatilis* L., and the life cycle requires only 2 hosts. Adult worms occur in the fish's intestine and gravid females release eggs that are voided with the faeces. Larvae develop in the hemocoel of the isopod *Asellus aquaticus* L. (Andryuk, 1974), and fish become infected by preying on isopods harbouring the infective cystacanth stage.

Previous studies on adult *A. lucii* have investigated the parasite's development and morphology (Andryuk, 1974, 1981), distribution in the host gut (Serov, 1985), seasonal occurrence (Komarova, 1950; Andryuk, 1974; Andersen, 1978; Mishra, 1978; Priemer, 1979), and fecundity (Serov, 1984). However, there are no detailed accounts of the population biology of this species, based on examination of adult and larval stages collected from the same habitat. A previous paper described the life history and population dynamics of larval *A. lucii* in *A. aquaticus* from the Forth and Clyde canal in Glasgow, Scotland (Brattey, 1986). The present paper describes the life history and population biology of adult *A. lucii* in fishes collected from the same locality.

MATERIALS AND METHODS

Sampling

Samples of perch, pike (*Esox luscius* L.), and roach (*Rutilus rutilus* L.) were collected during May 1979–September 1981 from the Forth and Clyde canal at Temple, Glasgow, Scotland (grid ref. NS 550694) with the aid of a beam trawl, gill nets of various mesh sizes (9–40 mm), seine nests, or by angling. No other fish species were observed in the study area. Fish were placed in plastic bags labelled with the date and location of capture, and taken to the laboratory on ice in an insulated cooler. All fish were frozen and stored at −20 C.

Examination of perch

Perch were thawed and their fork lengths measured to the nearest millimetre. The sex of each fish was noted and the age determined by examining an opercular bone (Le Cren, 1947). Stomach fullness was assessed visually using a points system (Craig, 1978) where 0 = empty, 1 = some food in the pyloric region, 2 = some food in the pyloric and cardiac regions, 3 = pyloric region full and some food in the cardiac region, and 4 = full stomach. Dietary items were sorted and the percentage occurrence of *A. aquaticus* determined. Specimens of *A. aquaticus* were dissected and the number of cystacanths of *A. lucii* recorded. The liver, mesenteries, spleen, kidney, and gonads of approximately 20% of the perch were also examined for *A. lucii*. The remainder of the alimentary tract, including the pyloric caeca, was examined and specimens of *A. lucii* were removed, counted, and sexed. Female worms were teased open and according to the degree of develop-

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* Present address: Department of Fisheries and Oceans, P.O. Box 5667, St. John's, Newfoundland, Canada A1C 5X1.

TABLE I. *Definitive hosts of* Acanthocephalus lucii *in the Forth and Clyde canal, Scotland.*

Details of infection	*Perca fluviatilis* (n = 525)	*Esox lucius* (n = 19)	*Rutilus rutilus* (n = 6)
Prevalence	79.4	63.2	33.3
Abundance ± SE (max.)	8.1 ± 0.68 (119)	10.3 ± 4.29 (68)	1.3 ± 0.99 (6)
No. of males	1,896	77	3
No. of females	2,355	119	5
% Immature	16.7	42.9	80.0
% Developing	34.7	53.8	20.0
% Gravid	48.6	3.4	0

ment of the shelled acanthors assigned to 1 of 3 stages: (I) immature—with ovarian balls only; (II) developing—with a mixture of ovarian balls and immature shelled acanthors; (III) gravid—with mature shelled acanthors. Male worms complete their development in the intermediate host (Brattey, 1986); hence different stages could not be distinguished.

Examination of pike and roach

Pike and roach were examined in a similar manner to perch, except that ages were not determined and stomach fullness was not assessed, although dietary items were identified when possible.

Statistics

The terms prevalence (% infected) and abundance (mean number of parasites per fish) follow the recommendations of Margolis et al. (1982). Differences in these infection parameters between monthly samples and fish age classes were statistically evaluated using *G*-tests and *t*-tests or the Student–Newman–Keuls (SNK) test, respectively. Raw data for the *t*- and SNK-tests were $\log_{10}(x + 1)$ transformed before analysis. Bartlett's test and the Scheffé–Box test confirmed that the sample variances were homogeneous after transformation. Dispersion patterns of *A. lucii* among perch were investigated initially by chi-square analyses of the ratio of the mean number of parasites per fish ($\bar{x}$) to the sample variance (s^2). Observed frequencies of *A. lucii* were then fitted to 4 discrete 2-parameter distributions: Neymann Type A, Polya-Aeppli, negative binomial, and Poisson lognormal. These distributions differ in their degree of relative skewness, the Neymann Type A being the least skewed and the Poisson lognormal the most skewed. The distributions were fitted using maximum likelihood methods (Ross, 1980), and cells in each distribution were grouped in a consistent manner to minimize the frequency of 0 counts. Relative goodness of fit to each model was tested by comparing the value of the log-likelihood ratio statistic G^2 (Table V).

TABLE II. *Ages of perch,* Perca fluviatilis, *collected from the Forth and Clyde canal, Scotland.*

Sampling date	Number in each age class 1+	2+	3+	4+	5+
May 1979	14	8	1	0	0
July	1	21	1	0	1
September	0	6	18	0	0
October	0	15	12	0	0
January–March 1980	2	6	5	1	0
April	0	10	7	1	1
May	0	15	4	1	0
June	0	0	27	2	0
July	0	0	38	0	0
August	0	0	40	1	0
September	0	0	40	1	0
October	0	0	30	0	0
November	0	1	31	1	0
December	0	5	48	2	0
January 1981	0	3	54	2	0
February	0	2	25	2	0
March	0	0	19	0	0

RESULTS

Adult *A. lucii* were found in the alimentary tract of perch, pike, and roach (Table I). Although there were no significant differences in the prevalence ($P > 0.1$) or abundance ($P > 0.05$) of *A. lucii* between perch and pike, perch were the most abundant fish species in the canal, comprising >95% of the total number of fish collected.

Female *A. lucii* were significantly more abundant than males in perch and pike (chi-square test, $P < 0.001$), but not in roach, which were rare and yielded few specimens. Gravid females were observed only in perch and pike, although they were also observed in roach from the canal during a previous study (Brattey, 1979). Nonetheless, the high proportion of gravid females in perch combined with the high relative abundance of this fish species indicates that perch were the most important definitive hosts for sustaining *A. lucii* in the Forth and Clyde canal.

Relationship between perch age and *A. lucii* infection

Perch ages ranged from 1+ to 5+, although most samples consisted of fish in the 2+ or 3+ age classes and the population appeared to be dominated by individuals from the 1978 year class (Table II). Few fish were caught during January–March 1980, therefore, samples for this period were pooled. Perch fork lengths ranged from

TABLE III. *Occurrence of* Acanthocephalus lucii *in relation to the age of perch,* Perca fluviatilis, *from the Forth and Clyde canal, Scotland. Prevalences or abundances not sharing the same superscript are significantly different* (P < *0.05).*

Details of infection	Age class 1+ (n = 17)	2+ (n = 92)	3+ (n = 400)	4 and 5+ (n = 16)
Prevalence	76.5[a]	72.8[a]	80.3[a]	100.0[b]
Abundance ± SE	6.3 ± 1.88[a]	6.9 ± 1.26[a]	8.7 ± 0.79[a]	15.9± 5.05[b]
Maximum	27	56	119	83
Number of female *A. lucii*	58	314	1,820	123
% Immature	32.8	18.3	53.2	28.5
% Developing	58.6	53.2	30.0	43.9
% Gravid	8.6	28.5	53.7	52.0

8.0 to 23.4 cm, but most were between 10 and 15 cm.

All age classes of perch harboured *A. lucii,* but data for 4+ and 5+ perch were pooled due to small sample sizes (Table III). The prevalence and abundance of the parasite did not differ significantly ($P > 0.05$) among age classes 1+, 2+, and 3+, but older perch were more heavily infected. Immature, developing, and gravid worms were found in all perch age classes. Older perch tended to have a higher proportion of gravid worms but these fish comprised only 3.2% of the total number of perch examined and they were well distributed among the samples; therefore, it is unlikely that seasonal trends in the occurrence of *A. lucii* (see below) were due to heterogeneity in the age composition of the perch samples.

Fish diet, feeding activity, and recruitment of *A. lucii*

Stomach content analysis indicated that perch in the canal fed throughout the year (Table IV). Mean stomach fullness was variable (0.3–2.6) but there was a seasonal trend in feeding activity. Perch stomachs contained more food during late spring and summer and least food during mid-winter. The most common food items (percent occurrence) were Chironomidae (25.7), microcrustacea (20.0), larval Trichoptera (16.6), larval *Sialis* (14.3), and *A. aquaticus* (7.8). Fish were rare in perch stomachs (1.1). Perch appeared to feed on *A. aquaticus* throughout the year. Although the percentage occurrence of the isopod in perch stomachs was low (≤18.4%), there was a seasonal trend in isopod intake. Percentage occurrence was significantly higher (*G*-test, $P < 0.05$) during May–September than during October–March.

The stomachs of all infected pike (12 of 19) contained perch but no isopods, which suggests that pike acquired their worm burdens secondarily from infected perch.

A total of 213 *A. aquaticus* was recovered from the stomachs of 525 perch. The distribution of isopods among perch stomachs was highly aggregated (s^2: $\bar{x} > 1$, $P < 0.001$; Fig. 1A) and 1 stomach contained 73 isopods, or 34.3% of the total. Forty-one (19.3%) of the isopods recovered were infected with cystacanths of *A. lucii.* Infected isopods were found in most months (Table IV), which suggests that recruitment of cystacanths occurred throughout the year. The distribution of cystacanths among perch stomachs was also aggregated (s^2: $\bar{x} > 1$, $P < 0.001$; Fig. 1B) and 1 stomach contained 13 cystacanths, or 34.1% of the total. Most infected isopods harboured 1

TABLE IV. *Stomach fullness and percentage occurrence of* Asellus aquaticus *in the stomachs of perch,* Perca fluviatilis, *from the Forth and Clyde canal, Scotland. Stomach fullness was assessed visually using a points scale from 0 to 4 (after Craig, 1978).*

Sampling date	Sample size	Mean stomach fullness	Percentage occurrence of *A. aquaticus*
May 1979	23	2.4	8.3*
July	24	1.8	4.2*
September	24	1.8	0
October	27	1.3	7.4*
January–March 1980	14	0.3	7.1*
April	19	1.6	0
May	20	2.6	10.0*
June	29	2.4	10.3
July	38	1.4	18.4*
August	41	0.9	12.2*
September	41	1.5	14.6
October	30	1.0	6.7*
November	33	1.1	12.1
December	55	1.5	0.2*
January 1981	59	0.4	3.9*
February	29	0.6	6.9*
March	19	2.3	5.3

* Months when isopods harbouring cystacanths of *A. lucii* were found in perch stomachs.

cystacanth; the maximum number per isopod was 3.

The percentage occurrence of *A. aquaticus* in perch stomachs did not differ significantly between perch age classes 1+ (5.9), 2+ (8.7), and 3+ (7.0). However, in 4+ and 5+ perch the percentage (20) was significantly higher (*G*-test, $P < 0.05$), which suggests that *A. aquaticus* was a more important dietary item to older perch.

Seasonal occurrence of *A. lucii* in perch

Parasite population size: Infected perch were found throughout the year, but the prevalence and abundance of *A. lucii* differed significantly between monthly samples ($P < 0.01$ in each instance) and during consecutive years there was an annual cycle in the size of the parasite population (Fig. 2A). Prevalence was generally high (70–90%) during May–September in both years, but declined through autumn and winter and reached a minimum (55%) in January–March. Abundance was highest (14–16 worms per fish) in May in both years, but decreased during subsequent months to a minimum (2–3 worms) in January–March.

Parasite population structure: The structure of the adult *A. lucii* population was generally heterogeneous (Fig. 2B). Immature worms (stage I) were found in most months, which suggests that recruitment of cystacanths occurred throughout the year. Most of the female cystacanths ingested during early spring reached the developing stage during April–May and by June many were gravid; therefore, maturation appeared to coincide with higher water temperatures (see Brattey, 1986). The proportion of gravid females remained high (51–83%) during June–December, but diminished during January–March (21%) as the population died out. Shelled acanthor production was not measured directly, but most shelled acanthors were probably produced during summer and autumn (June–November) when the size of the parasite population and the proportion of gravid females were highest.

The proportion of immature females increased during January–March 1981, suggesting that recruitment of a subsequent generation of adult parasites began early in winter and overlapped with the few remaining gravid females from the preceding generation. In general, the data in Figure 2A and B suggest that there was 1 generation of adult *A. lucii* per year in the canal, although

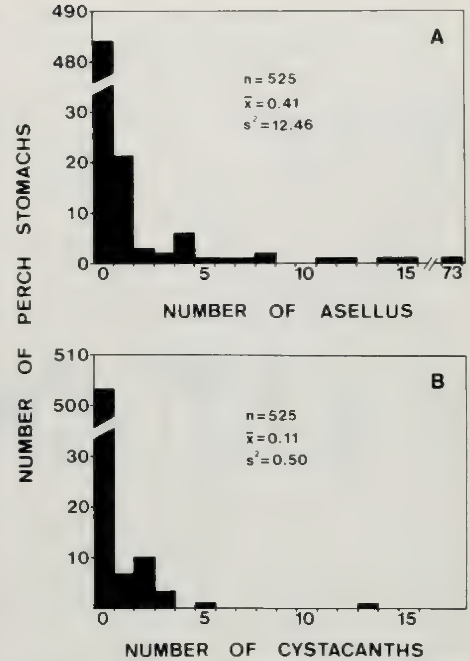

FIGURE 1. Frequency distributions of (**A**) *Asellus aquaticus* and (**B**) cystacanths of *A. lucii* in the stomachs of perch from the Forth and Clyde canal, Scotland. n = Sample size, s^2 = sample variance, and $\bar{x}$ = arithmetic mean.

each generation tended to overlap and there was little synchrony in the development of individuals within each generation.

Sex ratio: There was a distinct seasonal change in the sex ratio of *A. lucii* in perch (Fig. 2C). During spring and summer, male and female worms were present in approximately equal numbers; however, during autumn there was a progressive change in the sex ratio in favour of females and by winter the ratio of females to males was close to 2:1.

Frequency distribution: To investigate seasonal changes in the distribution of *A. lucii* among perch, frequency distribution histograms were constructed after grouping data for April 1980–March 1981 into six 2-mo periods (Fig. 3). In each of the six distributions the ratio of the sample variance (s^2) to the mean ($\bar{x}$) was significantly ($P < 0.001$) greater than unity indicating an aggregated distribution.

The negative binomial generally fitted the data better than the other models (lowest G^2 values), but the Poisson lognormal was the best model

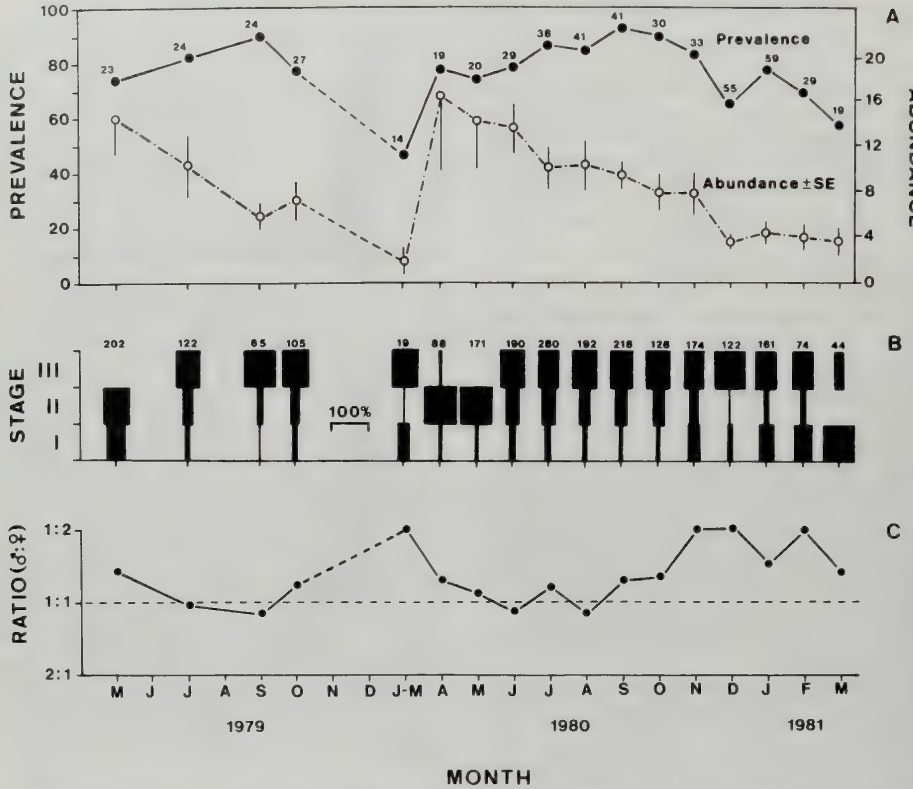

FIGURE 2. Seasonal occurrence of *A. lucii* in perch from the Forth and Clyde canal, Scotland. (**A**) Prevalence and abundance ± SE. Numbers next to points are sample sizes. (**B**) Population structure. Immature (stage I), developing (stage II), and gravid (stage III) females are shown as a proportion of the total number of females recovered each month. Totals are indicated above each histogram. (**C**) Sex ratio (males: females).

for data for December–January 1981 (Table V). The values of the exponent k of the negative binomial were all less than 1 indicating that the degree of parasite aggregation was generally high; however, k increased consistently during April–October, suggesting a decrease in aggregation through summer and autumn.

DISCUSSION

This study shows that there was a pronounced seasonal cycle in the numbers of adult *A. lucii* in perch from the Forth and Clyde canal. Perch ingested cystacanths throughout the year; therefore, there was a continuous flow of *A. lucii* through the perch population and the overall numbers of the adult parasite were constantly in a state of flux.

Annual population cycles are common among intestinal helminths of freshwater fishes (see Chubb, 1982) and they have been attributed to seasonal changes in (i) availability of infective larvae, (ii) resistance of hosts to infection, and (iii) host feeding behaviour (Kennedy, 1975; Anderson, 1976). The cycle observed here cannot be attributed to a seasonal appearance of larvae because cystacanths of *A. lucii* are present throughout the year (Brattey, 1986). Changes in host resistance also seem an unlikely cause of the cycle because prevalence increased during May–September (Fig. 2A) and immature worms were found in most months (Fig. 2B), suggesting that perch remain susceptible to infection throughout the year. The general correlation between changes in the percentage occurrence of *A. aquaticus* in perch stomachs and the overall numbers of adult *A. lucii* suggests that the annual cycle was mainly due to changes in perch feeding behaviour.

Acanthocephalus lucii apparently has 1 gen-

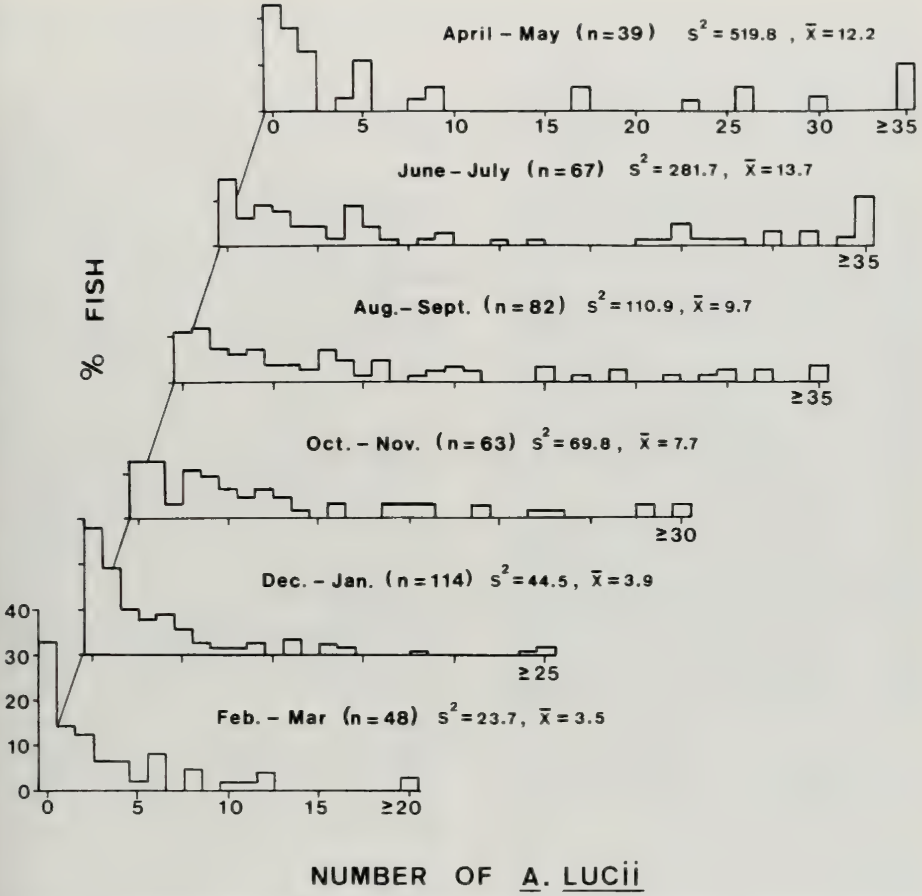

FIGURE 3. Seasonal changes in the frequency distribution of *A. lucii* in perch from the Forth and Clyde canal, Scotland. n = sample size, s^2 = sample variance, and $\bar{x}$ = arithmetic mean.

eration per year in the Forth and Clyde canal. Although the development of individuals within each generation was not synchronous, the life history for the majority of individuals in the population can be summarized as follows: adult worms produced most shelled acanthors during summer and fall, and gradually diminished in numbers during winter. Larvae developed in *A.*

TABLE V. *Maximum likelihood estimates for parameters of various frequency distribution models fitted to data on the distribution of* Acanthocephalus lucii *in perch,* Perca fluviatilis, *from the Forth and Clyde canal, Scotland. Sample sizes are indicated in Figure 3.*

Date	Degrees of freedom	Neymann Type A			Polya-Aeppli			Negative binomial			Poisson lognormal		
		M1	M2	G^2	M1	Q	G^2	μ	k	G^2	M	σ	G^2
April–May	14	1.98	5.08	98.95	1.26	0.88	38.14	11.30	0.37	22.62	1.04	1.94	20.76
June–July	14	2.28	5.84	87.88	1.68	0.88	30.28	13.94	0.60	22.56	1.78	1.52	28.22
August–September	13	2.31	4.23	65.01	1.86	0.81	17.29	10.21	0.78	6.66	1.68	1.26	8.26
October–November	12	2.08	3.59	30.80	1.84	0.76	11.53	7.72	0.89	7.34	1.50	1.14	8.93
December–January	14	1.40	2.82	73.38	1.14	0.71	28.83	4.03	0.56	12.64	0.57	1.33	7.58
February–March	10	1.27	2.83	22.36	1.05	0.72	11.40	3.81	0.51	8.88	0.45	1.49	9.81

aquaticus during summer and fall. Some larvae reached the cystacanth stage during late summer or fall; others became dormant during winter and completed development the following spring (Brattey, 1986). A few cystacanths were ingested by perch during winter, but most recruitment of adults occurred during spring and summer. These worms matured and produced shelled acanthors during late spring, summer, and fall, thereby completing the life-cycle and initiating the next generation. The life history of *A. lucii* in other habitats is difficult to determine because other published accounts lack data on the seasonal development of larval stages, or the recruitment and maturation of the adults. Nonetheless, some populations appear to exhibit overlapping annual generations similar to that reported here (Komarova, 1950; Wierzbicki, 1970; Andryuk, 1974; Andersen, 1978).

Although gravid females were observed in most months, *A. lucii* exhibited a seasonal maturation cycle that coincided with higher water temperatures (see Brattey, 1986) during spring and summer. Other field studies of *A. lucii* have reported similar findings (Komarova, 1950; Andersen, 1978) and data obtained from experimental infections support the hypothesis that maturation of the parasite is controlled by temperature. In perch maintained at 19 and 12 C, *A. lucii* will develop shelled acanthors in approximately 3 and 7 wk, respectively. However, at 5 C development is almost completely inhibited and shelled acanthors are not fully developed even after 13 wk (Brattey, 1982). Host hormonal changes associated with spawning, rather than water temperature, may be involved in controlling maturation of some species of helminth parasite (Kennedy, 1969, 1975; Wootten, 1974; Amin, 1978). However, maturation of *A. lucii* cannot be controlled by the host's reproductive hormones because gravid female *A. lucii* were recovered from sexually immature perch. Also, in the laboratory *A. lucii* will produce shelled acanthors when reared in sexually immature rainbow trout, *Salmo gairdneri*, or in adult perch that are not in spawning condition (Brattey, 1982).

The present study reveals that the sex ratio of *A. lucii* changes as the parasite population ages. The sex ratio of the adults was initially 1:1 (Fig. 2C), but the subsequent increase in the proportion of females indicates that females of *A. lucii* generally survive longer than males in the intestine of the definitive host. This trend is consistent with that shown by other acanthocephalan species (Burlingame and Chandler, 1941; Awachie, 1966; Crompton and Whitfield, 1968; Crompton et al., 1984).

The increase in the prevalence and abundance of *A. lucii* with perch age agrees with the pattern observed in other populations (Andersen, 1978; Mishra, 1978; Lee, 1981). In perch from the Forth and Clyde canal the increase cannot be attributed to the parasite accumulating over successive years because individual worms did not survive in perch for more than 1 yr. The rarity of fish in perch stomachs suggests that the trend was not due to cannibalism with transfer of worms from young to old fish. Stomach content analysis suggests that the higher infection levels reflect an age-related increase in the relative importance of *A. aquaticus* in the perch diet. Other field studies have also shown that benthic invertebrates such as *Asellus* spp. often become increasingly important in the diet of older perch (McCormack, 1970; Ali, 1973; Craig, 1978; Rask and Hiisivuori, 1985).

A notable finding was the highly aggregated distribution of *A. lucii* among perch. Although various mechanisms can generate such dispersion patterns (Crofton, 1971; Anderson, 1974; Keymer and Anderson, 1979; Anderson and Gordon, 1982; Crompton et al., 1984), the aggregated distribution of *A. lucii* was at least partly due to heterogeneity in perch feeding behaviour (Fig. 1). The latter, when combined with the clumped distribution of cystacanths among intermediate hosts (Brattey, 1986) and selective predation on infected isopods (Brattey, 1983) would result in a highly aggregated input of larvae into the perch population.

The mechanisms responsible for seasonal changes in the degree of parasite aggregation are more difficult to identify. The decrease in aggregation through summer and autumn, when prevalence remained high (Fig. 2), suggests that some form of density-dependent parasite mortality was operating. However, the present study does not provide unequivocal evidence that regulatory mechanisms were acting on the adult *A. lucii* population. Studies on other acanthocephalan species suggest that regulation of population size may be achieved by processes such as intraspecific competition or partial immunity acting on parasite establishment or survival (Burlingame and Chandler, 1941; Holmes et al., 1977; Uznanski and Nickol, 1982; Crompton et al., 1984; Brown, 1986). Detailed experimental investigations would be necessary to determine whether

similar control mechanisms operate on *A. lucii* populations in perch.

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MAST CELL RESPONSES TO *HYMENOLEPIS MICROSTOMA* INFECTION IN MICE

Marie Novak and Susan Nombrado
Department of Biology, University of Winnipeg, Winnipeg, Manitoba, Canada R3B 2E9

ABSTRACT: Murine mast cells (MC) responded strongly to *Hymenolepis microstoma* infection. Starting day 7 postinfection (PI) and continuing until the end of the experiment (35 days PI), significantly larger numbers of MC were present in both the duodenum and bile duct of infected mice than in uninfected controls. In animals challenged with 5 cysticercoids 2 wk after primary infection, the MC response in the duodenum, but not in the bile duct, was of even greater degree than in naive hosts. The majority of MC in the duodenum of infected and challenged mice were intraepithelial mucosal MC, whereas in the bile duct the majority were connective tissue MC. Hypertrophy of the duodenal submucosa and of the bile duct wall was noticeable in all infected and challenged hosts. Worms in primary infections were not affected by the host response, but none of the worms in the challenge dose became established. It is postulated that the type of MC involved in specific immune response of the host is the intraepithelial MC, whereas the cell type participating in general inflammatory events is the connective tissue MC.

In primary infections of mice, *Hymenolepis microstoma* is not normally rejected (Moss, 1971; Howard et al., 1978), whereas the number of secondary worms and their growth decrease as either the intensity or duration of primary infection increases (Howard, 1976). The growth of worms is retarded most during the first 4 days of reinfection, but once the worms reach the bile duct, the growth rate becomes similar to that of the primary infection (Howard, 1977). Using standard histological staining techniques, many authors have demonstrated that *H. microstoma* elicits a strong host response (Dvorak, 1963; Bogitsh, 1966; Moss, 1971; Simpson and Gleason, 1975; Pappas, 1976; Pappas and Mayer, 1976; Pappas and Schroeder, 1977; Novak et al., 1985). The histopathological changes were always obvious in the bile duct but not in the duodenum.

New evidence is showing that the intestinal mucosal mast cells (MMC) are functionally active during the expulsion process in nematode infections (Befus and Bienenstock, 1979; Befus et al., 1979; Woodbury et al., 1984; Lee et al., 1986). Because these cells were also found in increased numbers in intestinal walls of hosts infected with tapeworm *H. diminuta* (Andreassen et al., 1978), we became motivated to study the possible role played by these cells in the inflammatory and rejection processes in tapeworm infections. The present experiment was designed to examine the responses of MC in the duodenum and bile duct of a mouse host infected and challenged with *H. microstoma,* and to observe if the inflammatory changes in the intestine differ from those in the bile duct.

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MATERIALS AND METHODS

Swiss-Webster male mice, 6 mo old, were divided into 3 groups: uninfected controls, infected (primary infection), and reinfected (secondary infection) groups. Mice were infected by stomach tube with 5 cysticercoids of *Hymenolepis microstoma* each. Reinfected mice received 5 more cysticercoids each 2 wk later. Five animals were sacrificed from the primary and uninfected groups on days 7, 14, 21, 28, and 35 postinfection (PI). In the secondary group, 5 mice were killed on days 21, 28, and 35 PI, which corresponded to days 7, 14, and 21 of the secondary infection. The worms were dissected out of the bile duct and duodenum, counted, and their dry weights determined by drying them on preweighed aluminum dishes for 24 hr in an oven at 80 C. Bile ducts and 4-cm portions of duodenum were fixed in Carnoy's fluid for 6 hr. Tissues were embedded in paraffin, sectioned at 6 μm, and stained by the method of Cheema and Scofield (1985).

Lamina propria MMC, intraepithelial MMC, and connective tissue MC (CTMC) were counted in both the duodenum and bile duct, following morphological and staining criteria of Enerback (1966), Rahko (1970), Befus and Bienenstock (1979), and Jarrett and Haig (1984). In the duodenum, the cell types were counted in the region of 10 villous-crypt units (VCU) per mouse as defined by Miller and Jarrett (1971). The cells were also counted in the submucosa, muscularis externa, and serosa in that region. In the bile duct, the cells were counted in 3-mm lengths of each sample. Cell counts were made under $\times 250$ magnification. The thicknesses of intestinal and bile duct walls were measured in all groups. Total and differential MC counts, the numbers and dry weights of worms, and thickness of tissue samples are presented as arithmetic means. Statistical significance between the groups was analyzed using Student's t-test (Zar, 1984).

TABLE I. *Numbers and dry weights of* H. microstoma *recovered from infected and reinfected mice.*

Days PI	Group	No. of worms per mouse (mean ± SE)	P	Dry weight of worms (mg) (mean ± SE)	P
7	Infected	3.8 ± 0.5		0.1 ± 0.0	
14	Infected	3.4 ± 1.1		19.6 ± 5.4	
21	Infected	3.4 ± 0.4	NS*	92.2 ± 10.9	NS*
	Reinfected	3.8 ± 0.7		76.3 ± 15.3	
28	Infected	3.6 ± 0.9	NS	111.4 ± 15.2	NS
	Reinfected	3.6 ± 0.8		103.0 ± 22.0	
35	Infected	3.4 ± 1.0	NS	97.4 ± 15.7	NS
	Reinfected	4.4 ± 0.4		92.3 ± 3.2	

* NS = nonsignificant.

RESULTS

Numbers and dry weights of worms

The numbers of *H. microstoma* recovered from infected and reinfected mice and their dry weights are presented in Table I. All primary worms recovered on day 7 were already present in the bile duct. On the average, 3.5 worms were recovered from infected mice, whereas reinfected mice had about 3.9 worms. These differences were not statistically significant. In the reinfected group no small secondary worms were found either in the duodenum or bile duct on day 7 (day 21 of primary infection). Thus, it can be assumed that they were expelled from the host before this time. The worms in the infected group gained weight rapidly until day 28 PI, with a slight decline in their weight on day 35. The worms recovered from reinfected mice on days 21, 28, and 35 weighed slightly but not significantly less than those from the infected group.

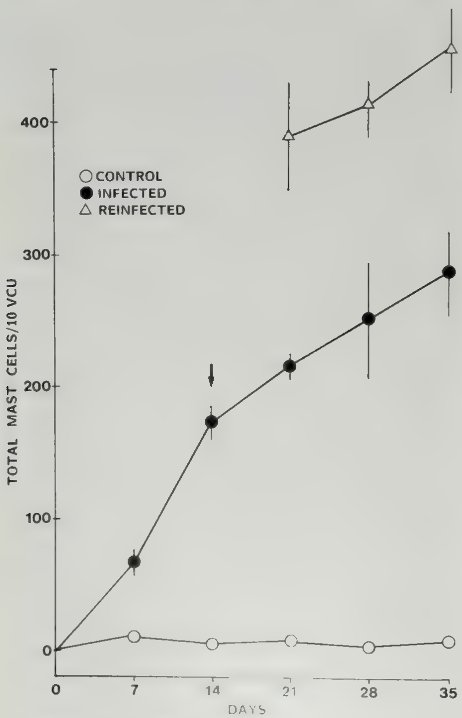


FIGURE 1. Total MC counts ± SE per 10 VCU of duodenum of uninfected (controls) and *H. microstoma*-infected and reinfected (arrow) mice.

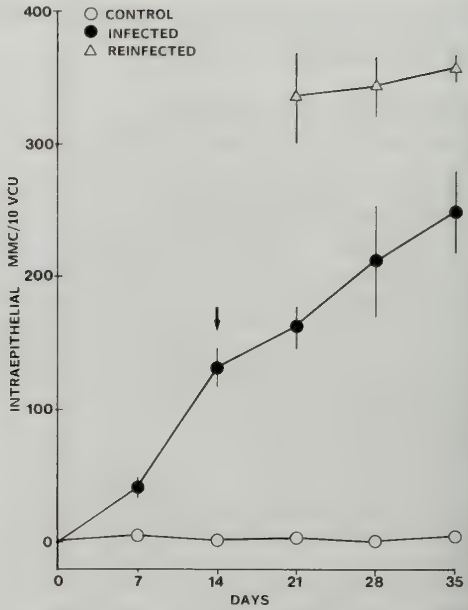


FIGURE 2. Intraepithelial MMC counts ± SE per 10 VCU of duodenum of uninfected (controls) and *H. microstoma*-infected and reinfected (arrow) mice.

Duodenal MC

Very few MC were seen in the duodenum of uninfected mice (Fig. 1). They were mostly CTMC, found in the vicinity of blood vessels in the submucosa. However, in the infected mice the total number of duodenal MC increased dramatically as the infection progressed, and their numbers nearly doubled in reinfected hosts. All differences were highly significant ($P \leq 0.001$). The majority of these duodenal MC in infected and reinfected hosts were intraepithelial MMC (Fig. 2). The other 2 subpopulations, lamina propria MMC and CTMC, were in the minority (Fig. 3a, b). The duodenal lamina propria MMC increased slowly, but significantly, in numbers in

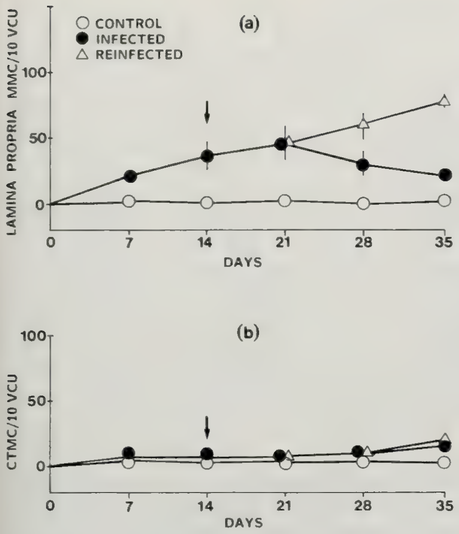


FIGURE 3. **a.** Lamina propria MMC counts ± SE per 10 VCU of duodenum of uninfected (controls) and *H. microstoma*-infected and reinfected (arrow) mice. **b.** CTMC counts ± SE per 10 VCU of duodenum of uninfected (controls) and *H. microstoma*-infected and reinfected (arrow) mice.

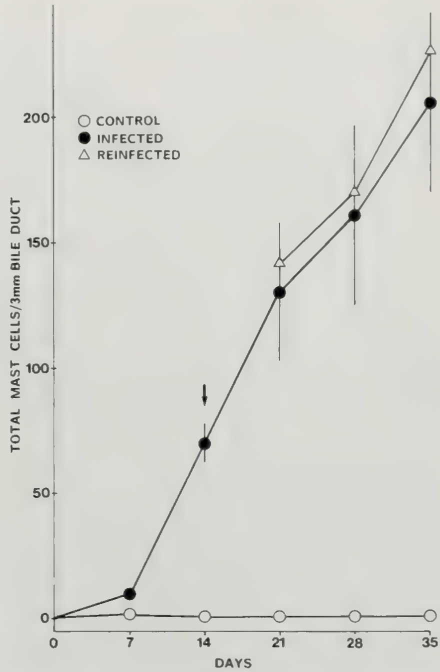


FIGURE 4. Total MC counts ± SE per 3 mm of bile duct of uninfected (controls) and *H. microstoma*-infected and reinfected (arrow) mice.

infected mice from day 7 to 21 PI and then declined (Fig. 3a). In reinfected mice, the numbers of lamina propria MMC were not significantly different from those in the infected group 1 wk after the challenge, but they were significantly higher on days 28 and 35 of the experiment. There was no increase in the numbers of CTMC between uninfected and infected mice on days 7 and 14, or between infected and reinfected mice on days 21, 28, and 35 PI (Fig. 3b). However, on days 21, 28, and 35 of the experiment, significant differences in CTMC numbers were obtained among uninfected, infected, and reinfected groups.

Bile duct MC

Bile ducts of uninfected mice contained very few MC (Fig. 4). Bile ducts of infected mice, on the other hand, already contained significantly higher numbers of MC on day 7 of infection and thereafter they increased exponentially in numbers until the end of experiment. There was an even greater increase in the total MC numbers in the reinfected group, but this increase was not statistically different from that of the primary-infected group. Intraepithelial MMC did not appear in the primary-infected bile duct during the first 21 days of the experiment (Fig. 5a). Then, on days 28 and 35, they were found to have increased significantly in numbers. In the reinfected group, the response of these cells seemed to be more rapid, but there were no significant differences in cell numbers between primary and secondary infections. Lamina propria MMC responded faster than the intraepithelial population. A significant increase in these cells was already seen in primary-infected mice by day 7 and it continued until day 35 PI. In the challenged mice, the lamina propria MMC decreased slightly in numbers, but not significantly from those in primary-infected hosts. The majority of MC seen in the bile duct wall of infected and reinfected mice were the CTMC (Fig. 6). Although the CTMC responded faster and were more numerous in challenged mice, their numbers did not differ significantly from those in the primary infection.

Thickness of the duodenum and bile duct

When the total thickness of the duodenal wall was measured, the infected duodenum was sig-

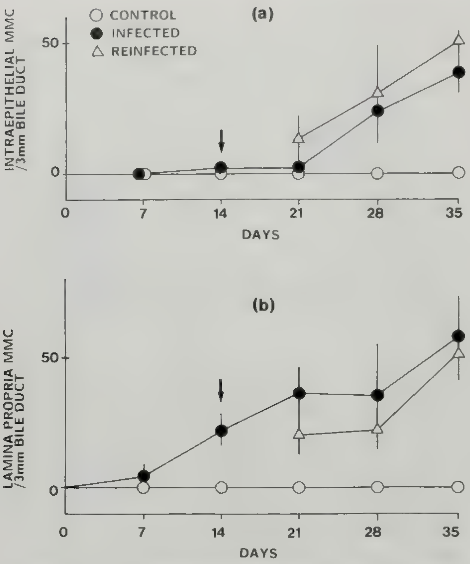


FIGURE 5. **a.** Intraepithelial MMC counts ± SE per 3 mm of bile duct of uninfected (control) and *H. microstoma*-infected and reinfected (arrow) mice. **b.** Lamina propria MMC counts ± SE per 3 mm of bile duct of uninfected (control) and *H. microstoma*-infected and reinfected (arrow) mice.

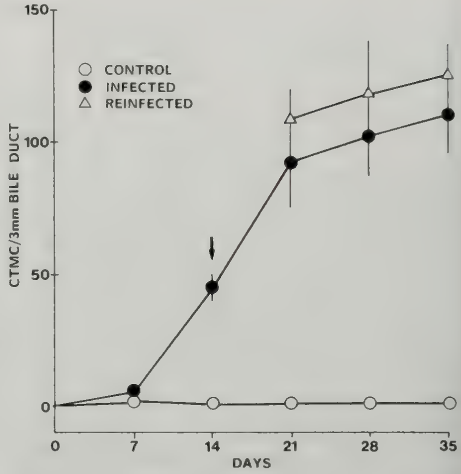


FIGURE 6. CTMC counts ± SE per 3 mm of bile duct of uninfected (control) and *H. microstoma*-infected and reinfected (arrow) mice.

nificantly thicker than the uninfected control only on day 35 PI. The duodenum of reinfected mice became significantly thicker earlier, on day 28, or 2 wk after reinfection (Table II). There was no significant difference in the total thickness of the duodenal wall between the infected and reinfected groups. The measurements of individual tunicas revealed no difference in the thickness of tunica mucosa and tunica muscularis externa plus serosa in control, infected, and reinfected groups, though the villi in the latter 2 groups became highly convoluted (Fig. 7; Table III). However, the tunica submucosa became significantly thicker by day 14 of the primary infection and stayed thicker until day 35. The submucosa of reinfected duodenum got even thicker, but not significantly more, than that of the infected group. The MMC together with CTMC were seen in larger numbers in the thickened submucosa from day 28 PI.

The bile duct wall was thicker in both primary- and secondary-infected mice by day 7 PI and

TABLE II. *Thickness (μm) of duodenal and bile duct wall in uninfected (control), infected, or reinfected mice with* H. microstoma.

Days PI	Group	Total intestinal wall (μm) (mean ± SE)	P			Total bile duct wall (μm) (mean ± SE)	P		
7	Control	690.0 ± 30.0	NS*			66.2 ± 3.1	0.002		
	Infected	724.6 ± 34.8				211.2 ± 29.5			
14	Control	694.0 ± 22.9	NS			61.1 ± 6.9	0.001		
	Infected	745.0 ± 37.0				380.4 ± 37.3			
21	Control	665.8 ± 26.5	NS	NS		53.0 ± 5.7	0.001	0.001	
	Infected	696.0 ± 16.3			NS	573.1 ± 31.9			NS
	Reinfected	698.6 [illegible] 31.2				612.0 ± 42.4			
28	Control	675.6 ± 16.[illegible]	NS	0.001		49.2 ± 4.8	0.001	0.001	
	Infected	740.0 [illegible]			NS	655.7 ± 37.4			NS
	Reinfected	833.2 [illegible]				670.8 ± 49.2			
35	Control	[illegible] ± 4.8	NS	0.001		51.2 ± 7.9	0.001	0.001	
	Infected	[illegible]			NS	664.0 ± 44.7			NS
	Reinfected	[illegible]				786.4 ± 111.4			

* NS = nonsignificant.

TABLE III. *Thickness (µm) of individual tunicas of duodenum in uninfected (control), infected, or reinfected mice with* H. microstoma.

Days PI	Group	Mucosa (µm) (mean ± SE)	P		Submucosa (µm) (Mean ± SE)	P			Muscularis externa + serosa (µm) (mean ± SE)	P		
7	Control	570.0 ± 30.0	NS*		55.22 ± 7.7	NS			52.8 ± 4.8	NS		
	Infected	593.8 ± 25.4			76.5 ± 13.0				54.3 ± 6.5			
14	Control	578.4 ± 6.4	NS		57.6 ± 6.7	0.001			58.0 ± 23.1	NS		
	Infected	582.4 ± 36.7			120.2 ± 7.2				42.8 ± 7.1			
21	Control	569.0 ± 32.7	NS	NS	56.8 ± 4.3	0.001	0.001		40.0 ± 7.4	NS	NS	
	Infected	556.0 ± 25.4			100.8 ± 21.1			NS	35.6 ± 5.4			NS
	Reinfected	547.6 ± 33.6			113.5 ± 20.3				37.8 ± 11.7			
28	Control	584.0 ± 23.8	NS	NS	55.6 ± 3.7	0.001	0.001		36.0 ± 6.1	NS	NS	
	Infected	613.2 ± 38.3			87.0 ± 4.4			NS	39.8 ± 13.6			NS
	Reinfected	637.2 ± 17.4			124.8 ± 6.1				46.4 ± 14.0			
35	Control	583.6 ± 21.7	NS	NS	56.2 ± 7.8	0.001	0.001		64.0 ± 14.1	NS	NS	NS
	Infected	644.4 ± 23.1			136.8 ± 4.8			NS	34.8 ± 11.4			
	Reinfected	644.0 ± 39.4			140.4 ± 7.9				45.0 ± 4.9			

* NS = nonsignificant.

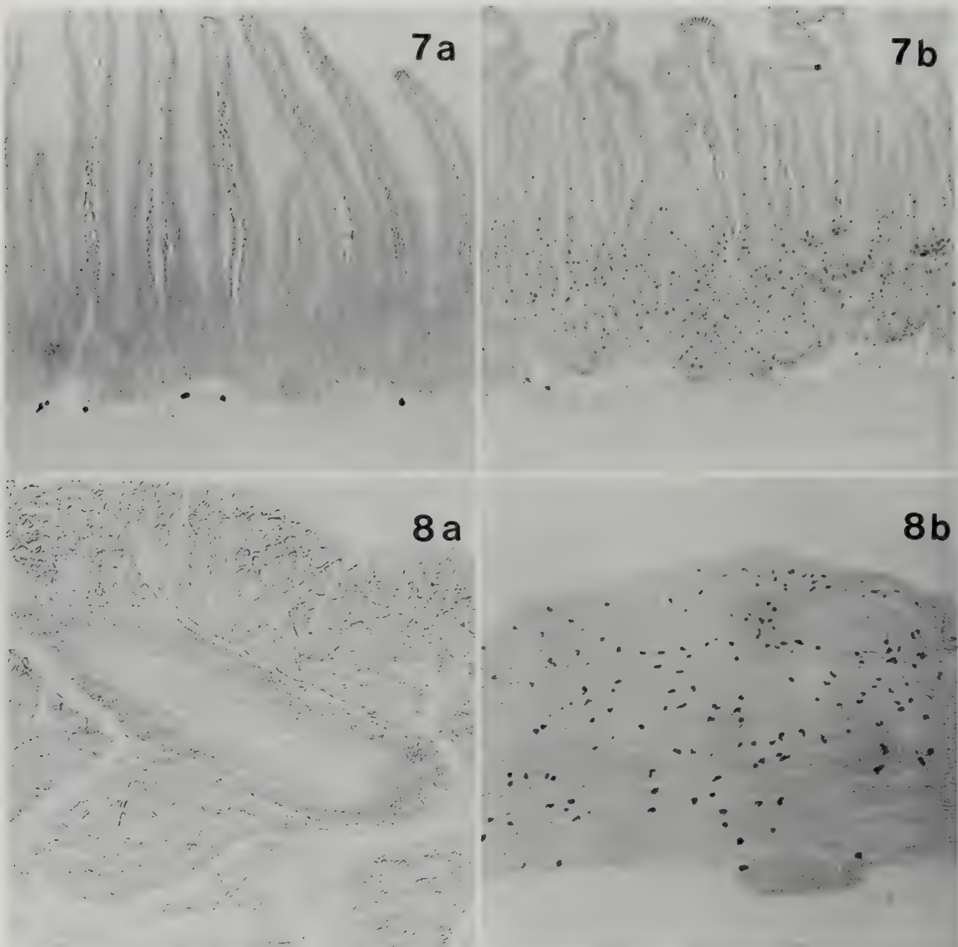
stayed thicker than that of the uninfected control until the end of the experiment (Table II). In challenged mice the bile duct became thicker than that in the primary-infected group, but this difference was not statistically significant. Many CTMC and some MMC were seen in the connective tissue region of the hypertrophied bile duct (Fig. 8).

DISCUSSION

This study showed that a pronounced mastocytosis developed in the duodenum and the bile duct of mice in response to infection with *H. microstoma.* A similar increase in the numbers of intestinal MC was reported for *Nippostrongylus brasiliensis* (Miller and Jarrett, 1971; Kelly and Ogilvie, 1972; Befus and Bienenstock, 1979; Befus et al., 1979; Nawa and Korenaga, 1983), *Trichinella spiralis* infections in rats (Woodbury et al., 1984), mice (Tronchin et al., 1979), and humans (Gustowska et al., 1983), and *H. diminuta* in mice (Andreassen et al., 1978) and rats (Hindsbo et al., 1982).

It has been established that 2 distinct MC types, CTMC and MMC, exist. They differ in their origin, morphology, granule content, and staining properties (Crowle and Phillips, 1983; Jarrett and Haig, 1984). In an infected rat, the MMC are seen in both the lamina propria and the epithelium, whereas in a murine host they appear almost exclusively in an intraepithelial location. This is also true for *H. diminuta*-infected mice (Andreassen et al., 1978) and in our experiment for mice infected with *H. microstoma.* In nematode infections the interleukin 3-releasing specific T blasts, as well as gut MMC and their precursors, all disappear very rapidly when the local antigenic stimulation abruptly stops—i.e., after the worms are rejected (Guy-Grand et al., 1984). The decline in numbers of MMC was also observed in mice following expulsion of *H. diminuta* (Andreassen et al., 1978). Since the primary worms in *H. microstoma*-infected mice are not rejected, and therefore continue to stimulate the MMC proliferation and transformation, it is not surprising that in the present study the duodenal MMC kept increasing in numbers until the end of the experiment. The specific role of MC in the expulsion of intestinal worms is not fully understood. It has been suggested that some of the MC products may participate in the rejection process. Enerback (1986) demonstrated that high levels of histamine, in blood and intestine, correlated with the proliferative MMC response in rats infected with *N. brasiliensis,* and Woodbury et al. (1984) showed that the systemic secretion of rat MMC protease II coincided with the immune expulsion of both *T. spiralis* and *N. brasiliensis.* Furthermore, the rat MMC were found to contain cytoplasmic IgE, in addition to bearing surface IgE (Lindsay et al., 1983; Jarrett and Haig, 1984; Lindsay and Williams, 1985) and the cells showed antigen reactivity (Lee et al., 1985). Although the direct killing of helminth parasites by MC could not be demonstrated, they dramatically increased the killing effect of eosinophils (Capron et al., 1978). Mediators of this cooperation, leucotrienes, were detected in significantly increased levels in the intestines and blood of rats challenged with worm antigen (Moqbel et al., 1986).

As our results showed, mastocytosis also de-



FIGURES 7, 8. **7a.** Control (uninfected) duodenum; note absence of MMC in tunica mucosa and 2 CTMC in thin tunica submucosa. **b.** Reinfected duodenum (35 days PI). Duodenal villi are convoluted and numerous MMC are located mostly in the crypts epithelium. Two CTMC (large, dark cells) and few MMC (small, lighter cells) can be seen in swollen submucosa. **8a.** Control (uninfected) bile duct; MC are absent. **b.** Reinfected bile duct (35 days PI). Note the hypertrophy of bile duct wall and the presence of many CTMC (large, dark cells) and some MMC (small, lighter cells). All tissues were fixed in Carnoy's and stained with alcian blue–safranin. ×100.

veloped in the bile duct, though it did not seem to influence the rejection of *H. microstoma.* All primary worms present in the bile duct on day 7 PI survived until day 35 PI. Other workers studying the growth and development of this cestode in naive mice reported similar observations (Moss, 1971; Howard, 1976). Howard et al. (1978) suggested that survival of primary *H. microstoma* could be due to the fact that the scolex of a worm, located inside the bile duct, was protected from immune changes occurring in the small intestine. The presence of low numbers of MMC in the bile duct wall of infected mice in our experiment offers another possible explanation. The majority of MC seen in the *H. microstoma*-infected bile duct were CTMC. Biochemical findings showed that these cells contain large amounts of histamine and heparin, the latter product not found in the MMC (Jarrett and Haig, 1984). Both histamine and heparin play a

role in general inflammatory reactions by increasing blood flow and vascular permeability of blood vessels, resulting in the recruitment of various leucocytes to the site of infection (Lee et al., 1986). Direct correlation between the hypertrophy of bile duct wall and duodenal submucosa, and CTMC hyperplasia in *H. microstoma*-infected mice supports the view of the above functions of these cells.

It was observed by Howard (1976) that although host immunity to *H. microstoma* is capable of rejecting or of stunting the growth of a secondary infection, this immunity appears to do no harm to a primary infection. He postulated that the decrease in growth of a secondary infection with an increased intensity of duration of primary infection is a result of the increased amount of antigen presented to the immunologically primed host's cells. In our study all secondary *H. microstoma* worms were rejected by day 7 of a challenge. The strong secondary response of MMC in these mice indicates that the secondary worms probably lived in the duodenal lumen for some time (but less than 7 days) before they were expelled.

Thus, it seems that expulsion of the intestinal tapeworms and the presence of antigen-reactive intraepithelial MMC might be connected events. As shown in the present experiments and by Andreassen et al. (1978), both secondary *H. microstoma* and primary and secondary *H. diminuta* are rejected from intestines of murine hosts that are able to generate intraepithelial MMC response. On the other hand, a rat host, in which *H. diminuta* is not normally rejected, is unable to generate an intraepithelial globule–leucocyte response (Hindsbo et al., 1982). However, although the intraepithelial MMC subpopulation seems to contribute to rapid expulsion of the worms, it does not seem to be the sole effector involved in this process. As shown by Andreassen et al. (1978), nude mice that do not contain MC of any kind, but are able to mount worm-specific antibodies, also expel *H. diminuta,* although more slowly than normal mice. This would indicate that, at least in the case of *H. diminuta* in a mouse, the worm's antigens induce both T-dependent and T-independent immune responses.

Clearly, further work is required to define functional characteristics of the MC subpopulations. New improved methods for isolation of MC of high purity levels from homogeneous *in vivo* mucosal sources (Befus et al., 1982; Lee et al., 1985) should provide more opportunity to study the form and function of MMC from local sites of infection in various host–parasite systems. Subsequently, the interaction of MC types with other cell types involved in helminth infections needs to be analyzed in order to expand our knowledge of how the cells in the immunological web interact.

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HOMOLOGOUS AND HETEROLOGOUS RESISTANCE OF *ECHINOSTOMA REVOLUTUM* AND *E. LIEI* IN ICR MICE

Donald W. Hosier, Bernard Fried*, and Jason P. Szewczak
Department of Biology, Moravian College, Bethlehem, Pennsylvania 18018

ABSTRACT: To study resistance of echinostomes in the mouse, female ICR mice were challenged homologously or heterologously with *Echinostoma revolutum* or *E. liei* metacercariae. Mice challenged homologously had significantly fewer worms which weighed less than those from control mice. In heterologous studies where the primary infection was not eliminated with an anthelmintic, the number of worms in challenged mice was not significantly different than that in controls which received only the primary infection. However, the mean dry weight worm of the secondary infection was less than that of controls. Mice challenged with *E. revolutum* 2 days after a 21-day-old *E. liei* infection was eliminated with Zanil contained significantly fewer *E. revolutum*, which weighed less than those of controls.

Albino mice infected with *Echinostoma revolutum* developed resistance to a homologous challenge infection (Sirag et al., 1980; Christensen et al., 1984, 1986), but did not develop heterologous resistance against *Schistosoma mansoni* or *Fasciola hepatica* (Sirag et al., 1980). However, Christensen et al. (1981) showed that primary infection of *E. revolutum* in mice enhanced a challenge infection of *S. mansoni* compared to mice receiving only the primary infection.

The purpose of our study was to determine if ICR mice infected with *E. revolutum* or *E. liei* would develop resistance against the homologous or heterologous echinostome species.

MATERIALS AND METHODS

Encysted metacercariae of *E. revolutum* (North American strain; Beaver, 1937) and *E. liei* (Egyptian strain; Jeyarasasingam et al., 1972) from experimentally infected *Biomphalaria glabrata* snails were fed via stomach tube to 7–8-wk-old outbred, female ICR mice (Hosier and Fried, 1986). At primary or secondary exposure each mouse received 25 cysts. Each experimental group consisted of 10 mice except G and H which each had 20. At necropsy, mice were killed by cervical dislocation, worms were removed from the small intestine, placed in Locke's solution, and counted. Weights were determined by drying worms for 48 hr at 60 C.

In studies on the homologous resistance of *E. revolutum*, A, B, and C were exposed (Table I). Mice in A and B were killed 10 and 21 days postexposure, respectively. At 21 days postexposure C was secondarily exposed and D primarily exposed. Ten days later C and D were killed and adult worms counted.

In studies on the homologous resistance of *E. liei*, E, F, and G were exposed (Table II). To eliminate the primary infection on day 21, F and G were fed Zanil (340 mg/kg body weight) by stomach tube. On day 23, E and F were killed, and G was secondarily exposed. H, used as a drug residue control, was treated with Zanil on day 21 and primarily exposed on day 23. Group I was also primarily exposed on day 23, and on day 33 G, H, and I were necropsied.

In heterologous resistance studies in which *E. revolutum* was the primary exposure and *E. liei* the secondary exposure, J, K, and L each received a primary exposure (Table III). At 21 days postexposure, L received a secondary exposure and M a primary exposure with *E. liei*; J and K were necropsied at 10 and 21 days postexposure, respectively, and L and M were necropsied on day 31 of the experiment.

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* Department of Biology, Lafayette College, Easton, Pennsylvania 18042.

TABLE I. *Recovery data in ICR mice given a primary exposure of 25 metacercariae mouse with* E. revolutum *followed by 25 metacercariae of* E. revolutum *21 days later.*

Group	Primary infection	Secondary infection	Mean no. ± SE of worms recovered in days postinfection: 10	21	31	Mean dry weight/worm (mg)
A	*E. revolutum*	—	8.0 ± 2.3			N.D.*
B	*E. revolutum*	—		0		
C	*E. revolutum*	*E. revolutum*			1.7 ± 1.6	0.21
D	*E. revolutum*†	—			13.6 ± 4.8	0.56

* Not determined

† This primary infection was given 21 days after A, B, and C were exposed to *E. revolutum*

TABLE II. *Recovery data in ICR mice given a primary exposure of 25 metacercariae/mouse of* E. liei *followed by 25 metacercariae of* E. liei *23 days later.*

Group	Primary infection	Secondary infection	Mean no. ± SE of worms recovered in days postinfection		Mean dry weight/worm (mg)
			23	33	
E	*E. liei*	—	21.5 ± 1.5		
F*	*E. liei*	—	0		
G*	*E. liei*	*E. liei*		10.0 ± 1.9	0.51
H*	*E. liei*†	—		17.2 ± 1.4	1.40
I	*E. liei*†	—		20.6 ± 1.6	0.85

* Treated with Zanil on day 21 postinfection.

† This primary infection was given 23 days after E, F, and G were exposed.

In heterologous resistance studies in which *E. liei* was the primary exposure and *E. revolutum* the challenge, N and O were primarily exposed (Table IV). O was secondarily exposed 21 days later at which time P received a primary exposure to *E. revolutum.* N was necropsied at 21 days, and O and P 10 days later.

In another heterologous resistance study the primary infection of *E. liei* was eliminated with Zanil (Q, R, S, Table V). S was secondarily exposed and T and U primarily exposed to *E. revolutum* 23 days later. Q and R were necropsied 23 days postexposure. Ten days later S, T, and U were necropsied.

The Student's t-test was used to analyze the difference between means and $P < 0.05$ was considered significant. Since dry weights of worms were determined as a group, they could not be analyzed statistically.

RESULTS

As seen in Table I, *E. revolutum* worms were recovered on day 10, but not on day 21 postexposure (A and B). Mice challenged homologously with *E. revolutum* (C) had significantly fewer worms than mice in challenge control D. Also, the average dry weight of worms from C was less than that of D.

Since *E. liei* worms were not rejected 21 days postexposure, mice were treated with Zanil to eliminate parasites (G and H, Table II). Mice challenged homologously with *E. liei* (G) contained significantly fewer worms than H. Zanil did not reduce the number of parasites in the challenge infection (H as compared to I). The mean dry weight of challenge worms (G) was 0.51 mg compared to 1.4 mg for H.

Mice given a primary infection of *E. revolutum* and a challenge infection of *E. liei* (L) had 8.4 ± 1.3 *E. liei* compared to 10.2 ± 1.2 worms in M (Table III). In this experiment 2 mice (K) contained *E. revolutum* that were not rejected by day 21 postexposure, but group L at 33 days contained no *E. revolutum.* The mean dry weight of the *E. liei* challenge worms (L) was 0.14 mg, which was less than the 0.27 mg of the controls (M).

Mice given a primary infection of *E. liei* and 21 days later a secondary infection of *E. revolutum* (O) had 13.6 ± 1.4 *E. liei* and 3.5 ± 1.5 *E. revolutum* at 31 days (Table IV). As seen in Table IV, on day 21 *E. liei* were recovered from a primary infection (N). At necropsy *E. liei* were 31 days old and *E. revolutum* 10 days old, and they were easy to distinguish from each other based upon size and morphology. The number of *E. revolutum* (7.8) in the challenge control group (P) was not significantly greater than *E. revolutum* in O. Average dry weights of *E. revolutum* recovered from the control (P) and heterologous groups (O) were 0.29 and 0.08 mg, respectively.

TABLE III. *Recovery data in ICR mice given a primary exposure of 25 metacercariae/mouse with* E. revolutum *followed by 25 metacercariae of* E. liei *21 days later.*

Group	Primary infection	Secondary infection	Mean no. ± SE of worms recovered in days postinfection			Mean dry weight/worm (mg)
			10	21	31	
J	*E. revolutum*	—	15.8 ± 1.2			0.56
K	*E. revolutum*	—		2.7 ± 2.0		1.00
L	*E. revolutum*	*E. liei*			8.4 ± 1.3*	0.14
M	*E.* [illegible]	—			10.2 ± 1.2	0.27

* All recovered worms [illegible]

† This primary infectio[illegible] me time as the secondary infection in L.

TABLE IV. *Recovery data in ICR mice given a primary exposure of 25 metacercariae/mouse with* E. liei *followed by 25 metacercariae of* E. revolutum *21 days later.*

Group	Primary infection	Secondary infection	Mean no. ± SE of worms recovered in days postinfection 21	31	Mean dry weight/worm (mg)
N	*E. liei*	—	17.4 ± 2.5		0.80
O	*E. liei*	*E. revolutum*		3.5 ± 1.5*	0.08
				13.6 ± 1.4†	0.78
P	*E. revolutum*‡	—		7.8 ± 1.9	0.29

* *E. revolutum.*

† *E. liei.*

‡ This primary infection was given at the same time as the secondary infection in O.

Mice challenged with *E. revolutum* 2 days after a 21-day-old *E. liei* infection was eliminated with Zanil contained 2.3 ± 1.1 worms (S), whereas the challenge control (T) had 11.1 ± 1.9 worms (Table V). Average dry weights of *E. revolutum* recovered from S and T were 0.12 and 0.28 mg, respectively.

DISCUSSION

Our results show that ICR mice can develop resistance against a homologous challenge infection with either *E. revolutum* or *E. liei.* After homologous challenge, established worm burdens of *E. revolutum* and *E. liei* were significantly reduced 87.5 and 42%, respectively.

Christensen et al. (1981) first described the echinostome they used as the Egyptian strain, *E. liei.* In subsequent papers, without justification (e.g., Sirag et al., 1980; Christensen et al., 1986), they refer to the same parasite as *E. revolutum.* In a personal communication to one of us (B.F.), Christensen stated that he now believes the parasite he works with is synonymous to the Egyptian *E. liei.*

Although Sirag et al. (1980) and Christensen et al. (1984, 1986) reported almost 100% resistance compared to our 42% reduction of *E. liei,* results of the 2 studies are not comparable since we used different exposure doses and mice strains.

Our results show for the first time that homologous resistance occurs in mice infected with the North American strain of *E. revolutum.*

Compared to challenge controls there was a 17% reduction in the worm burden of *E. liei* superimposed upon a primary infection of *E. revolutum* and a 55% reduction of *E. revolutum* superimposed upon a primary *E. liei* infection. These are not significant reductions, but the dry weight data suggest that conditions for growth of *E. revolutum* or *E. liei* were suboptimal in heterologous infections.

In the study where the primary infection with *E. liei* was eliminated with Zanil, there was a significant reduction (79%) in the number of challenge *E. revolutum* compared to challenge controls. The dry weight data confirm that *E. liei* can induce heterologous resistance against *E. revolutum* in ICR mice.

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TABLE V. *Recovery data in ICR mice given a primary exposure of 25 metacercariae/mouse with* E. liei *followed by 25 metacercariae of* E. revolutum 23 days later.

Group	Primary infection	Secondary infection	Mean no. ± SE of worms recovered in days postinfection 23	33	Mean dry weight/worm (mg)
Q*	*E. liei*	—	0		
R	*E. liei*	—	20.5 ± 3.2		0.90
S*	*E. liei*	*E. revolutum*		2.3 ± 1.1†	0.12
T*	*E. revolutum*‡	—		11.1 ± 1.9	0.28
U	*E. revolutum*‡	—		8.9 ± 2.2	0.26

* Treated with Zanil on day 21 postinfection.

† All recovered worms were *E. revolutum.*

‡ This primary infection was given 23 days after Q, R, and S were exposed.

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MOUSE SPLEEN CELL RESPONSES TO TRICHOMONAL ANTIGENS IN EXPERIMENTAL *TRICHOMONAS VAGINALIS* INFECTION

Peter R. Mason and Lovemore Gwanzura
Department of Medical Microbiology, University of Zimbabwe Medical School, P.O. Box A178, Avondale, Harare, Zimbabwe

ABSTRACT: Subcutaneous inoculation of live *T. vaginalis* into mice caused splenomegaly, particularly when using strains of parasites with low pathogenicity. The proliferative responses of spleen cells from uninfected mice, as measured by [^{3}H] TdR uptake, showed that trichomonal antigens, whether from strains with high or low pathogenicity, have no mitogenic activity. Spleen cells from mice infected with trichomonads of low pathogenicity showed a proliferative response to trichomonal antigens that was maximal after 4 days incubation. The proliferative response of spleen cells from mice infected with trichomonads of high pathogenicity continued for at least 6 days in the presence of the antigen. Moreover, in the latter case there was a significantly greater uptake of [^{3}H] TdR when cells were incubated with antigens of a highly pathogenic strain. These results support the view that although many antigens are common to strains with differing levels of pathogenicity, some antigens are more closely associated with strains that are more highly pathogenic. The strong proliferative response to these antigens may then be related to the clinical presentation of infection with these strains.

Studies on female patients infected with the sexually transmitted protozoan, *Trichomonas vaginalis,* have demonstrated a variety of immune responses, including the production of IgA in vaginal secretions (Ackers et al., 1975), circulating antibody in serum (Mason, 1979; Street et al., 1982), and increased responses of circulating lymphocytes to trichomonal antigens (Yano et al., 1983; Mason and Patterson, 1985). Clinically, infection may present as an acute vaginitis with a profuse discharge containing numerous degenerating phagocytes, or as a virtually asymptomatic infection (Fouts and Kraus, 1980). These differences are due in part to the potential for pathogenicity of the infecting strain. Early reports indicated a generally good correlation between clinical symptoms and pathogenicity as demonstrated by the subcutaneous mouse assay (Honigberg, 1961). Our experience in Zimbabwe also is that trichomonads isolated from women with a profuse vaginal discharge produce large lesions in mouse assays, whereas only small lesions are produced when strains from asymptomatic women are tested in the same way (Mason and Forman, 1982; Mason and Patterson, 1985).

Recent evidence has shown that pathogenicity of trichomonads may be correlated with surface antigens (Su-Lin and Honigberg, 1983), surface saccharides (Warton and Honigberg, 1983), and hemolytic activity of the parasite (Krieger et al., 1983). Alderete et al. (1986) have described an association between virulence for cell cultures and strains of *Trichomonas* that are deficient in a high molecular weight surface glycoprotein. It was suggested that the absence of such an immunogen may give protection from antibody-dependent protective mechanisms against them.

Apart from this suggestion, and evidence that it may influence chemotactic responses of phagocytes to trichomonal secretions (Mason and Forman, 1982), relatively little is known of the possible effect of the level of pathogenicity of *T. vaginalis* on immune responses by the host. In other protozoans, notably amebic (Diamantstein et al., 1980) and amebo-flagellate (Ferrante and Smythe, 1984) infections, pathogenic species have been found to have mitogenic activity for mouse T lymphocytes, a mechanism whereby these species may evade specific immune mechanisms of the host.

The experiments described in this paper were carried out to determine whether similar mechanisms occurred in experimental trichomoniasis and whether infection with a particular strain of *T. vaginalis* had an effect on the cell-mediated response to antigens of other strains.

MATERIALS AND METHODS

Antigen preparations

Strains of *T. vaginalis* were isolated from female patients and were maintained in agarless Diamond's medium as previously described (Mason and Forman, 1982). The pathogenicity of axenised cultures was determined by a modified mouse-inoculation assay (Mason and Forman, 1982). Strains were designated as having high pathogenicity (HP) if they produced a lesion of >100 mm^2 6 days following subcutaneous in-

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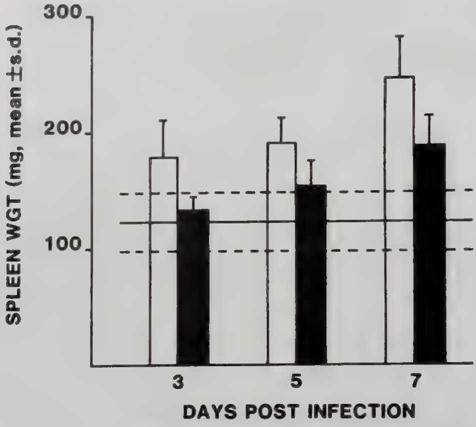

FIGURE 1. Splenomegaly in experimental murine trichomoniasis. Bars show spleen weights following subcutaneous infection with LP (unshaded) or HP (shaded) strains of *T. vaginalis* (n > 5 in each case). Horizontal lines show mean ± SD spleen weight of 10 uninfected mice.

oculation of mice with 10^6 live trichomonads. All of such strains were isolated from women showing clinical symptoms of acute trichomoniasis. Isolates from women with no overt symptoms, which produced small lesions in the mouse assay, were designated low pathogenicity (LP).

Overnight cultures of 1 HP and 1 LP strain were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS, 50 mM, pH 7.3), and resuspended in 3 ml PBS. Parasites were disrupted by subjecting the suspension to 3 freeze–thaw cycles (liquid N_2 −37 C waterbath), and the supernatants of centrifuged, disrupted cell preparations were filtered (0.2 μm pore filters, Millipore Corp., R.S.A.) to remove debris. Filtered supernatants were dialysed for 2 days against cold (4 C) distilled water, and the protein content was determined by the method of Lowry et al. (1951). Aliquots were freeze-dried for storage.

Mouse infections

These experiments used 4–6-wk-old mice bred in the University of Zimbabwe. For some experiments, mice were infected by inoculating 10^6 motile *T. vaginalis* in 0.5 ml Diamond's medium with 1% agar, subcutaneously. Control animals received 0.5 ml Diamond's with agar but without trichomonads. Spleens from such mice were removed 6–7 days postinfection.

Six different trichomonal isolates were used to infect the mice, 3 being HP and 3 being LP by the above criteria. Each of these strains was inoculated into at least 2 mice for spleen cell preparations.

Spleen cell preparation

Mice were killed by cervical dislocation, and spleens were removed aseptically. Adhering material was removed, without damaging the spleen capsule, before weighing the spleen. Spleen cells were collected by physical disruption of the spleen in 5 ml of sterile culture medium (RPMI-1640, Flow Labs., U.K., with 20 mM HEPES buffer pH 7.3, 100 U/ml penicillin, 100 μg/ml streptomycin). Debris was allowed to settle by gravity, and spleen cells remaining in suspension were decanted into sterile tubes, washed once with culture medium, and resuspended in culture medium with 15% fetal bovine serum (RPMI-FBS) at 10^7 methylene blue-excluding mononuclear cells per ml. Cultures were prepared as detailed below within 1 hr of spleen cell preparation.

Cultures

Spleen cell cultures were prepared in triplicate in 96-well sterile flat-bottomed microtiter plates (Flow Labs., U.K.). Cell suspensions (100 μl) were incubated with 100 μl of RPMI-FBS (as unstimulated controls); with 100 μl of antigen from HP or LP trichomonad strains (10 μg protein in RPMI-FBS); or with 100 μl phytohemagglutinin (PHA-P, Difco, U.S.A., dissolved in 5 ml water, diluted 1/128 with RPMI-FBS). Plates were incubated in 5% CO_2 : 95% air, at 37 C, for up to 6 days. Tritiated thymidine ([^{3}H] TdR, Amersham Radiochemicals, U.K., 1 μCi in 20 μl RPMI-FBS) was added for the final 18 hr of culture. Cells were harvested by semi-automated cell harvester onto glass-fibre discs and radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrophotometer.

Data analysis

Results were recorded as counts per minute (cpm) for each well. While the cpm for the triplicate cell preparations were consistent (lowest value always >80% of highest value), there was considerable variation between the cell preparations from different mice, even when comparing unstimulated cell cultures. It was therefore decided to use a stimulation index, rather than total cpm or differences in cpm between stimulated and unstimulated cells for comparison. The index was calculated as the cpm for stimulated culture/mean of the cpm's of the triplicate unstimulated cultures using cells from the same mouse. A stimulation index consistently >1.0 thus indicated spleen cell proliferation and was a reliable indication of response despite the variation between animals. Total counts have been included in the text, where relevant, for comparison with the index.

Results from replicates of the same experiment were pooled for calculation of mean and SD, and statistical analysis was by Student's *t*-test.

RESULTS

Spleen size

At 7 days postinfection spleens from infected mice were notably larger than from controls, particularly when mice were infected with LP trichomonads. Spleens from infected mice were significantly heavier than uninfected mice (Fig. 1) ($t = 10.3$, 6.8 for LP- and HP-infected mice vs. controls, $P < 0.01$) and spleens from LP strain-infected mice were significantly heavier than from HP strain-infected mice ($t = 4.2$, $P < 0.01$).

Spleens from mice infected for shorter periods

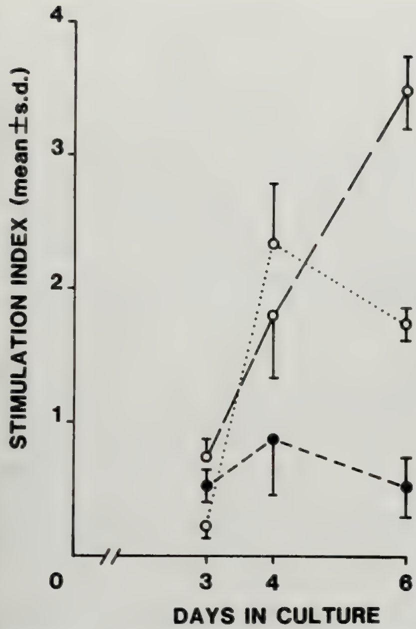

FIGURE 2. Proliferative response of mouse spleen cells to LP-strain antigen. Stimulation indices for cells from uninfected mice (●) and mice infected with LP (○·····○) or HP (○– – –○) trichomonads.

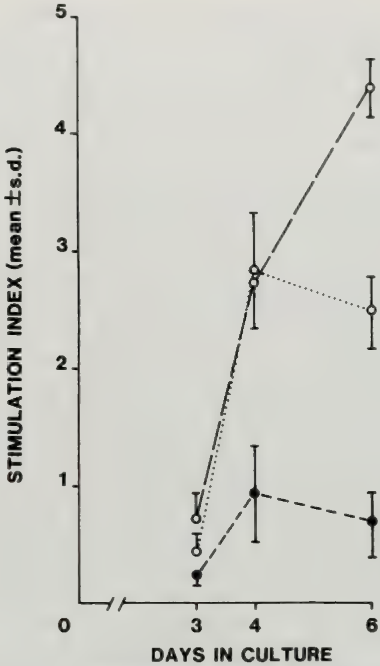

FIGURE 3. Proliferative response of mouse spleen cells to HP-strain antigen. Stimulation indices for cells from uninfected mice (●) and mice infected with LP (○·····○) or HP (○– – –○) trichomonads.

of time were also enlarged, in the case of LP infections significantly so at 3 days PI ($t = 4.9$, $P < 0.01$) and in the case of HP infections at 5 days PI ($t = 3.4$, $P < 0.01$).

Responses to antigens

Spleen cells from uninfected mice showed no proliferative response, as determined by [^{3}H] TdR uptake, when incubated for up to 6 days with trichomonal antigens. Actual cpm varied considerably from one cell preparation to another, with mean ± SD of cpm being 3,971 ± 3,285 to 5,161 ± 4,186 for 6-day incubations. Rarely however did the stimulation index exceed 1.0.

Spleen cells from mice infected for 6–7 days with LP strains of *T. vaginalis* showed increased [^{3}H] TdR uptake when incubated with either HP- or LP-strain antigens (Figs. 2, 3). In both cases maximal uptake was recorded after 4 days incubation and there was no significant difference in maximum cpm between cells incubated with HP and LP antigens (10,184 ± 4,523, 12,776 ± 1,942, respectively, $t = 1.29$).

Spleen cells from mice infected with HP trichomonads also showed a proliferative response when incubated with the antigens, though in this case maximal responses were recorded at 6 days incubation. There was a significantly greater uptake of [^{3}H] TdR by cells incubated with HP-strain antigen (19,706 ± 1,868 vs. 15,467 ± 1,873 for cells with LP-strain antigen, $t = 3.93$, $P < 0.01$) and the stimulation index was also significantly greater (4.41 ± 0.18 vs. 3.50 ± 0.25, $t = 8.83$, $P < 0.01$).

Spleen cells from both infected and uninfected mice showed increased [^{3}H] TdR uptake when incubated with PHA, with maximal responses in 48-hr cultures.

DISCUSSION

Urogenital infection with *T. vaginalis* may present with a spectrum of symptoms from virtually asymptomatic to a severe vaginitis with a copious purulent discharge and marked inflammation of the vaginal epithelium. It is now recognised that clinical presentation is a result of a number of factors, including parasite virulence and host response, both of which may change during the course of an infection. While we used

3 strains each of high and low pathogenicity to infect mice, we used only single strains to prepare antigens. There is some evidence that a culture population of *T. vaginalis* may be a heterogeneous mixture of more and less virulent trichomonads, and the expression of virulence by that culture may then vary in relation to the relative numbers of these cell types over a period of time (Alderete et al., 1986). Although the antigens we used were prepared from the same culture tubes used to infect the mice to determine pathogenicity, they may still have represented a heterogeneous mixture of trichomonad phenotypes.

Bearing this reservation in mind, we found no evidence that antigens from cultures with either high or low mouse pathogenicity levels had mitogenic activity for mouse spleen cells. This is in contrast to the mitogenic activity of other protozoan parasites such as *Entamoeba histolytica* (Diamantstein et al., 1980) or the pathogenic ameboflagellate *Naegleria fowleri* (Ferrante and Smythe, 1984). It was suggested that mitogenic activity by these pathogens, which was not a feature of closely related nonpathogenic species, was a mechanism whereby they could evade specific host responses. Since trichomoniasis in humans is essentially an infection restricted to the genital epithelium, evasion of such host responses may not be as essential to the survival of *T. vaginalis* as it is to more invasive organisms.

It should be noted that the genetic composition of the host may influence immunologic responses to trichomonal infection (Landolfo et al., 1980). Since the origin and breeding of the mice available to us for these experiments were uncertain, our conclusions must remain tentative until confirmed by studies using well-characterised mouse strains. Nevertheless, the continued proliferation of spleen cells from HP- but not LP-infected mice, the significantly greater response of such cells to HP antigen, and the significant differences in splenic enlargement of infected animals all point toward the view that major differences occur in the cell-mediated responses of animals sensitised by subcutaneous inoculation of live *T. vaginalis* of differing pathogenicity levels. Such experimental infections, it must be recognised, are radically different from natural infection with this parasite, and any extrapolation to possible events in human infections would be speculative. Indeed, previous studies using human peripheral blood lymphocytes found no differences in the response to antigens from different strains of *T. vaginalis* (Mason and Patterson, 1985), though the pathogenicity of the sensitising strain was not known and may, by chance, have been low in each of the patients studied.

The greater and more prolonged response of cells from HP-strain-infected animals in the present study may be explained by suggesting that some trichomonal antigens are shared by different strains of the parasite whereas others are characteristic of more highly pathogenic strains. Some support for this comes from quantitative immunofluorescence studies showing that more highly pathogenic strains have specific antigenic sites in their surface membrane in addition to antigens that are common to all strains (Su-Lin and Honigberg, 1983). A possible association between virulence for cell cultures and the array of specific immunogens at the trichomonal cell surface was also reported by Alderete et al. (1986), and the differences in spleen cell response to different strains that we have noted here presumably result from interactions between these surface immunogens and lymphocytes.

The functional significance of the spleen cell response is unclear, because cellular anti-trichomonal activity appears to be limited to cells of the polymorphonuclear (Rein et al., 1980) or monocyte–macrophage (Landolfo et al., 1980) lineage, at least *in vitro*. The presence of large numbers of these phagocytes, along with viable trichomonads, in vaginal discharge suggests they may be less effective *in vivo* and may in fact contribute to increased epithelial cell lysis. The greater sensitivity of lymphocytes to HP-strain antigens may then be a contributory factor in increased inflammatory responses to these strains.

We were not able, in these experiments, to determine the identity of the responding spleen cells, though the evidence from studies on human patients is that T lymphocytes respond to trichomonal antigens (Yano et al., 1983). Clearly, further studies into the nature of strain-related sensitisation, the characteristics of responding spleen cells, the influence of these on effector cells, and their role in protective immunity would add greatly to our understanding of the complex interaction between parasite and host in this infection.

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EXCYSTATION *IN VITRO* OF *ECHINOSTOMA LIEI* AND *E. REVOLUTUM* (TREMATODA) METACERCARIAE

Bernard Fried and Stefano Emili
Department of Biology, Lafayette College, Easton, Pennsylvania 18042

ABSTRACT: Metacercariae of *Echinostoma liei* and *E. revolutum* were excysted in an alkaline bile–trypsin medium at 41 C in the absence of acid–pepsin pretreatment. After 60 min at a pH of 7.8 or 8.0, excystation of *E. liei* reached 98%; optimal excystation of *E. revolutum* occurred at pH 8.2 and was 70% after 60 min. The rate of excystation was very rapid in *E. liei*, reaching 91% at 30 min, and less rapid in *E. revolutum* reaching 40% at 30 min. Almost 100% of the *E. liei* cysts stored for 5.5 mo at 4 C in Locke's 1:1 excysted in the medium, compared to 40% for *E. revolutum* treated identically.

Fried and Roth (1974) described a procedure for excystation of the metacercariae of *Parorchis acanthus* in an alkaline bile–trypsin medium in the absence of acid–pepsin pretreatment. As discussed by Fried and Ramundo (1987), this procedure has been used successfully to excyst 5 other digeneans.

The excystation of fresh and stored cysts of *Echinostoma revolutum* has been described (Fried and Butler, 1978; Fried and Perkins, 1982), but similar studies on the related species, *Echinostoma liei*, are not available. As discussed by Jeyarasasingam et al. (1972), differences in infectivity, physiology, and biochemistry may be helpful in distinguishing closely related echinostome species. The present study compares excystation in *E. liei* versus excystation in *E. revolutum*.

MATERIALS AND METHODS

Encysted metacercariae of *Echinostoma revolutum* and *E. liei* were maintained in laboratory infected *Biomphalaria glabrata* snails (Jeyarasasingam et al., 1972; Anderson and Fried, 1987) and dissected from the saccular kidney 1–28 days postencystment. They were excysted immediately after removal from snails (fresh cysts) or within 10 mo following storage at 4 C in Locke's 1:1 (stored cysts). There was no difference in excystation of fresh cysts used 1–28 days postencystment.

The excystation medium (Fried and Roth, 1974) contained 0.5% trypsin plus 0.5% bile salts in Earle's balanced salt solution adjusted with 7.5% $NaHCO_3$ to various alkaline pH values. To study excystation, 100 ± 2 cysts of each species were placed in a 30 × 15-mm petri dish containing 4 ml of the medium and maintained in an incubator at 41 ± 0.5 C. Only organisms completely free of all cyst walls were considered excysted.

To study the effects of pH on excystation, fresh cysts were placed in the unadjusted medium (pH 6.2) and in media adjusted to pH values of 7.0, 7.8, 8.0, 8.2, and 8.9. Three to seven trials were done at each pH for each species and the percent excystation was recorded after 60 min.

The rate of excystation was determined for both species using fresh cysts. Three to eight trials were run for each species and the percent excystation was recorded after 15, 30, 60, and 120 min in the medium maintained at pH 8.0 ± 0.2.

To examine the effects of storage, *E. revolutum* and *E. liei* were maintained at 4 C in Locke's 1:1 for up to 10 mo. The percent excystation at each storage period was determined for 100 cysts of each species following treatment in the medium at a pH of 8.0 ± 0.2 for 120 min.

RESULTS

Cysts of both species averaged about 150 μm in diameter. The outer cyst of *E. liei* (Fig. 1) was about one-half as thick as that of *E. revolutum* (see fig. 1 in Fried and Bennett, 1979). Excysted metacercariae of *E. liei* flattened under coverslip pressure were larger than those of *E. revolutum* (Figs. 2, 3). Excysted metacercariae of 10 *E. liei* fixed in hot alcohol–formalin–acetic acid averaged 270 μm in length × 70 μm in width compared to 250 × 70 μm for *E. revolutum*. The excretory concretions of *E. liei* were about 2–3 × larger than those of *E. revolutum* (Figs. 4, 5). During excystation, larvae of both species rotated rapidly within the cyst, breached the inner cyst, and disrupted the outer cyst wall prior to excystation (Fig. 6; see fig. 1 in Fried and Butler, 1978).

The effects of pH on excystation after 60 min in the bile–trypsin medium is shown in Figure 7. Excystation did not occur at pH 6.2 and was minimal at pH 7.0. Optimal excystation for *E. liei* occurred at pH 7.8–8.0 reaching 98%, and at pH 8.2 for *E. revolutum*, reaching 70%.

The rate of excystation is shown in Figure 8. It was very rapid for *E. liei*, reaching 91% by 30

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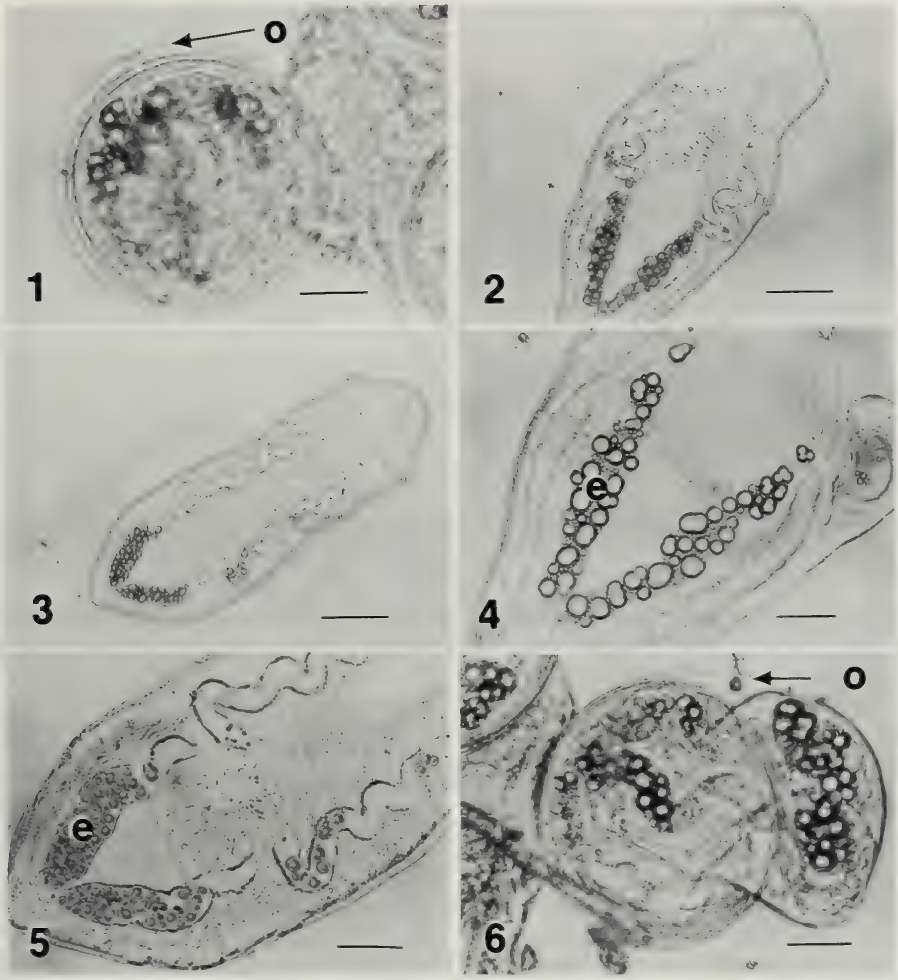

FIGURES 1–6. Photomicrographs of *Echinostoma liei* and *E. revolutum* metacercariae. **1.** Cyst of *E. liei*. Note outer cyst (o). Scale bar = 35 μm. **2.** Excysted metacercaria of *E. liei*. Scale bar = 80 μm. **3.** Excysted metacercaria of *E. revolutum*. Scale bar = 70 μm. **4.** Posterior end of *E. liei*. Note excretory concretions (e). Scale bar = 40 μm. **5.** Posterior end of *E. revolutum*. Note excretory concretions (e). Scale bar = 35 μm. **6.** Partially excysted *E. liei*. Note outer cyst (o). Scale bar = 36 μm.

min, and remained about the same for the next 90 min. For *E. revolutum* the rate increased rapidly to 40% by 30 min and less rapidly to 89% after 120 min.

The effects of cyst storage are shown in Figure 9. Fresh cysts did not excyst as well as those stored in Locke's 1:1 at 4 C for 2 wk. Almost 100% of the *E. liei* cysts stored for 5.5 mo excysted compared to about 40% for *E. revolutum* stored that long.

DISCUSSION

Subtle differences were noted in the morphology of encysted and excysted metacercariae of these related 37 collar-spined echinostomes, i.e., thickness of outer cyst walls, size of excretory concretions, and length of excysted metacercariae. Less subtle were the differences in excystation in the alkaline bile–trypsin medium. Both the rate and percent excystation of *E. liei* were

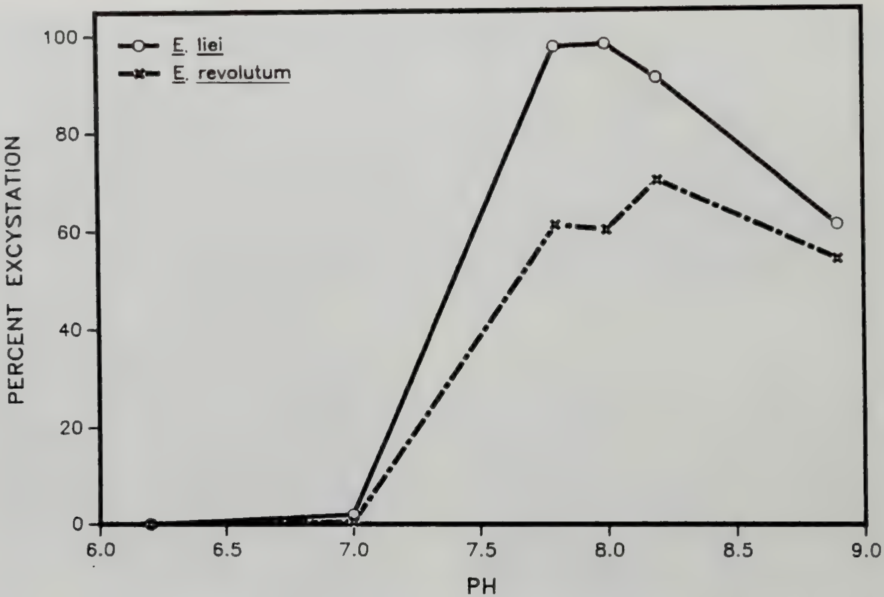

FIGURE 7. Percent excystation of *E. liei* and *E. revolutum* at various pH values in the alkaline bile–trypsin medium at 41 C for 60 min. Each symbol represents the mean of 300–700 cysts.

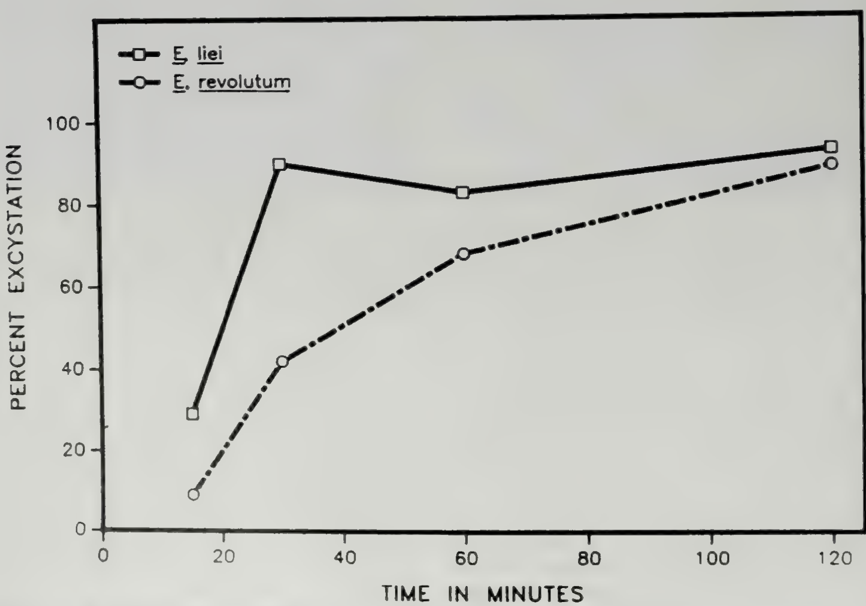

FIGURE 8. Percent excystation of *E. liei* and *E. revolutum* in the alkaline bile–trypsin medium at 41 C for 15–120 min. Each symbol represents the mean of 300–800 cysts.

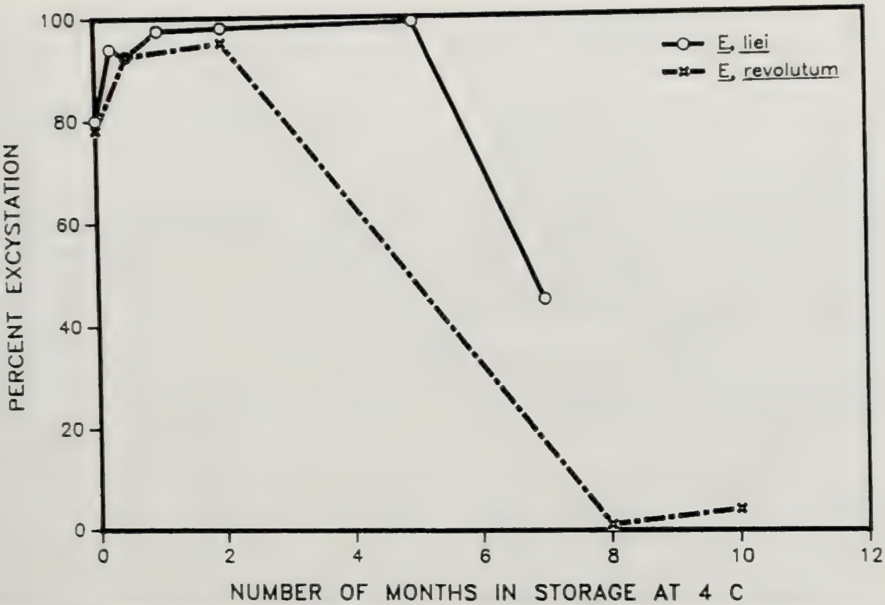

FIGURE 9. Percent excystation of *E. liei* and *E. revolutum* in the alkaline bile–trypsin medium at 41 C for 120 min following storage for up to 10 mo in Locke's 1:1 at 4 C. Each symbol represents the mean of 100 cysts.

considerably higher than in *E. revolutum*; the optimal pH for excystation of *E. liei* was slightly less alkaline than for *E. revolutum.*

Echinostoma liei is considerably more infective to various rodent hosts than is *E. revolutum* (Beaver, 1937; Jeyarasasingam et al., 1972; Christensen et al., 1980; Hosier and Fried, 1986). Probably, the rapid and high rate of excystation seen *in vitro* for *E. liei* also occurs *in vivo,* thus facilitating the establishment of this parasite in rodents. Comparative infectivity studies of *E. liei* and *E. revolutum* in avian hosts are lacking. Preliminary studies by one of us (B.F.) show that *E. liei* is considerably more infective to day-old domestic chicks than is *E. revolutum.*

Fresh cysts of both species did not excyst as well as those stored for several weeks. Dönges (1969) suggested that echinostome metacercariae require several days after encystment to become infective to a definitive host; the cysts are then capable of infecting a definitive host for 10–14 mo postencystment.

The very high percent excystation of stored cysts of *E. liei,* along with the ease of infecting laboratory hosts with this echinostome, make *E. liei* a more convenient model for studying experimental echinostomiases than *E. revolutum.*

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EIMERIA MAXIMA (APICOMPLEXA): A COMPARISON OF SPOROZOITE TRANSPORT IN NAIVE AND IMMUNE CHICKENS

Doone Riley and M. A. Fernando
Department of Pathology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

ABSTRACT: This study compared the early stages of infection in naive and immune chickens infected with *Eimeria maxima*. An immunoperoxidase stain was developed and used to detect sporozoites and early schizonts in tissue sections of intestinal epithelium. A significantly higher proportion of sporozoites was present in the crypts of naive chickens, 48 hr postinoculation of oocysts, compared to immune chickens. Sporozoites in immune birds tended to remain in the lamina propria rather than migrate to the crypts.

Sporozoites were found within intraepithelial lymphocytes (IEL's) in the epithelium, the lamina propria, and the crypts of both naive and immune chickens. Parasites in IEL's of immune birds at the ultrastructural level and there were no apparent morphological abnormalities.

Livers and spleens, of both immune and naive chickens that had been inoculated with *Eimeria maxima*, produced patent infections when fed to susceptible chickens. Infections could be transferred up to 72 hr postinoculation of the donor birds. Peak oocyst production in the recipient birds occurred 7–8 days after the transfers. This time period approximates the prepatent period in a natural infection and thus implies that the extraintestinal stage was a sporozoite.

Sporozoites of all 7 species of chicken coccidia enter the intestinal mucosa by penetrating villous epithelial cells. However, only 2 species, *Eimeria brunetti* and *E. praecox*, undergo further development at this site of entry (Pellerdy, 1974). Sporozoites of the other 5 species are transported to the crypt epithelial cells where they complete first-generation schizogony.

The transport of these sporozoites from villous to crypt epithelium has been studied by several groups. Van Doorninck and Becker (1957), Challey and Burns (1959), Patillo (1959), and Doran (1966) described sporozoites of *E. acervulina*, *E. necatrix*, and *E. tenella* in macrophages within the lamina propria of infected chickens and suggested that they were transported by macrophages. However, more recently, the mononuclear cells harbouring sporozoites of *E. maxima*, *E. necatrix*, and *E. tenella* have been identified ultrastructurally as intraepithelial lymphocytes (IEL) (Lawn and Rose, 1982; Al-Attar and Fernando, 1987; Fernando et al., 1987) and those with *E. acervulina* as having morphological characteristics of both IEL's and macrophages (Fernando et al., 1987). Surprisingly, sporozoites of *E. brunetti* and *E. praecox* were also found to be transported out of and back into villous epithelial cells by host mononuclear cells similar to those that transport *E. acervulina* (Fernando et al., 1987).

Results of work with *E. necatrix* (Al-Attar and Fernando, 1987), *E. acervulina*, *E. brunetti*, *E. maxima*, and *E. praecox* (Fernando et al., 1987) indicate that the transport of sporozoites is not confined to the mucosa as they have also been found extraintestinally.

The transport of sporozoites by intraepithelial lymphocytes and perhaps other mononuclear cells through the mucosa and within the peripheral circulation may be an important component in the immune response to these coccidia. In this paper we looked at differences in sporozoite transport between naive and immune chickens inoculated with large numbers of *E. maxima* oocysts. We examined the penetration of epithelial cells by sporozoites, their transport to the crypt and extraintestinal sites, and the development of first-generation schizonts. *Eimeria maxima* was chosen because it is the most immunogenic species infecting chickens (Lee and Fernando, 1978) and its sporozoites are transported by cells identified morphologically as IEL's.

MATERIALS AND METHODS

Animals

Chickens: The University of Guelph strain of white leghorns, raised coccidia-free in isolation facilities, was used in all experiments involving chickens. Chick starter containing 20% protein and no coccidiostats was used as feed (Shurgain, Floradale Feeds). Feed and water were provided *ad libitum* unless the chickens were to be inoculated with sporozoites or given tissue transfers. In these cases food was withheld for 2 hr prior to inoculation or transfer. Depending on the experiment, the age of the chickens used ranged from 2 to 8 wk.

Rabbits: Adult rabbits were obtained from Raymond's Rabbitry (St. Agatha, Ontario) and housed in the laboratory animal building at the University of Guelph. They were fed Purina rabbit pellets *ad libitum*

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and received tetracycline in their drinking water for 1 wk after arrival in the holding facilities.

Parasite

The Guelph strain of *Eimeria maxima* was used in all the experiments. Infection of chickens with oocysts was performed by oral inoculation, into the crop, using a sterile syringe and a 15-gauge udder infusion canula. Oocysts stored less than 4 wk were used for inoculations.

Detection of oocysts and estimation of oocyst numbers

Centrifugal flotations were used for the detection of oocysts and were done according to the method of Rose et al. (1984). Total oocyst production was estimated by measuring the numbers of oocysts in 24-hr collections of feces. The technique of Long and Rowell (1958) was used. Twenty-four-hour fecal collections were made from days 5 to 10 postinoculation of oocysts (PI) or posttransfer of tissues (PT).

Immunization of chickens

The chickens used in the experiments were classified as either immune or naive. The naive birds had been raised in isolation and were considered fully susceptible to infection with *E. maxima*. The immune birds had been inoculated at 5 wk of age with $10–20 \times 10^5$ sporulated oocysts of *E. maxima*. Oocyst production was monitored on days 5, 6, and 7 PI to ensure a patent infection had occurred. Naive and immune birds of the same age were used in any single experiment.

Rabbit anti-sporozoite sera

Eimeria maxima oocysts were isolated and purified by flotation in saturated sodium chloride. The oocysts were treated with sodium hypochlorite and broken to release the sporocysts by vibrating them at maximum setting for 15 sec with glass beads in a Mickle disintegrator (Brinkmann, New York). The suspension was sequentially sieved through 15- and 10-μm silkscreen cloth and the sporocysts in the filtrate were washed by centrifugation in phosphate-buffered saline pH 7.4 (PBS) at 1,000 *g* for 10 min. The sporocysts were suspended in excystation fluid (5% chicken bile and 0.25% trypsin in PBS) and incubated at 40 C for 45 min with gentle shaking. The excysted sporozoites were washed by centrifugation and resieved before purifying them using a metrizamide gradient (Wisher and Rose, 1984).

A total of 40×10^6 sporozoites in 4 ml were injected into each of 3 rabbits; half of the dose was injected intraperitoneally (i.p.) while the other half was emulsified in Freund's complete adjuvant (FCA) and injected subcutaneously (s.c.) in 4 sites (modified from Kuowenhoven and Kuil, 1976). Two weeks following injection the rabbits were bled by venipuncture of a lateral ear vein and the subcutaneous injections were repeated (incomplete Freund's adjuvant was used for these injections). Two weeks after the second injections the rabbits were sedated with Innovar Vet (fentanyl/droperidol; Pittman Moore), exsanguinated by cardiac puncture, and euthanized by intracardiac sodium pentobarbital injection.

Serum from inoculated rabbits was tested for specific activity against sporozoites by the dot-blot technique (Bio-Rad Immuno-Blot [GAR-HRP] Assay Kit, Bio-Rad Laboratories, California). This basically involves the passive transfer of sporozoites onto nitrocellulose paper followed by the application of the test antisera and the addition of an enzyme-linked anti-rabbit globulin antibody. The final step is the addition of the colour reagent which turns purple in the presence of the immune complex. The positive sera were divided into 0.5-ml aliquots and stored at −70 C.

Preparation of tissues for transfer to recipient chickens

Liver: After removing the gall bladder, the liver was chopped into small pieces and cold PBS was added to make a total volume of 30 ml. The mixture was homogenized in a blade type homogenizer (Sorvall Omnimixer) at a setting of 5–8 for 30 sec. Ten ml of the homogenate were removed and given to a recipient susceptible bird using a 12-cc disposable syringe and a 15-gauge udder infusion canula.

Spleen: The spleen was cut in half, placed in approximately 5 ml of cold PBS, and homogenized in a 15-ml Dounce homogenizer with a loose-fitting pestle (Kontes Biomedical Products, Vineland, New Jersey). Once the spleen was sufficiently homogenized to pass through the udder infusion canula (15–20 strokes) the homogenate was administered to a susceptible recipient bird as described for the liver transfer.

Small intestinal mucosa: The small intestine, from the distal end of the duodenum to Meckel's diverticulum, was removed and flushed with cold PBS. The intestine was cut open, placed on a glass plate, and the mucosa was scraped off using the edge of a glass slide. The mucosa was placed in a beaker and cold PBS was added to make a total volume of 10 ml. This was fed to a recipient bird in the same manner as the previous transfers.

Histology

Light microscopy: Liver, spleen, and intestine were fixed in Bouin's. Three areas of the intestine were routinely processed: (1) duodenum just distal to the pancreas, (2) jejunum midway between the pancreas and Meckel's diverticulum, and (3) jejunum at Meckel's diverticulum. Tissues were fixed in Bouin's for 12 hr, transferred into 70% alcohol, and embedded in paraffin at temperatures not exceeding 60 C. Sections (6–8-μm) were mounted on glass slides coated with Bondfast (Elmer's glue diluted with water). Slides were heated at 56–60 C for 30 min and then hydrated through xylene to 70% alcohol. Sections were stained with hematoxylin and eosin or with the immunoperoxidase technique.

Immunoperoxidase staining of the sections was performed using the Dakopatts DAKO PAP kit, system K548 (Dimensions, Missisauga), and the anti-sporozoite sera. The manufacturer's directions were followed, up to the stage of substrate addition. The slides were pretreated with 3% hydrogen peroxide and normal swine serum before incubating them for 20 min with rabbit anti-sporozoite serum diluted 1:500 or 1:200. The slides were then incubated with swine anti-rabbit globulin for 20 min, washed in 0.05 M Tris buffer, pH 7.6 (TRIS) and incubated in a peroxidase–antiperoxidase complex made in rabbits, then washed in TRIS. At this point diaminobenzidine tetrahydro-

chloride (DAB), available from Zymed Laboratories, San Francisco, was substituted for AEC substrate (Bourne, 1983; Wordinger et al., 1983). Slides were incubated with the DAB solution (0.25 ml of 0.01 M DAB in TRIS buffer was diluted in 4.75 ml TRIS buffer and 3 drops 3% hydrogen peroxide were added) for 10 min, rinsed with distilled water, dehydrated through alcohol, cleared in xylene, and mounted with DPX.

Electron microscopy: Samples were routinely taken from the jejunum midway between the pancreas and Meckel's diverticulum. Tissue sections for electron microscopy were either pinned out on rubber mats or flattened on glass slides with rubber bands, then washed with cold PBS before being immersed in fixative containing 1% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The fixed tissue was cut into pieces 1–2-mm square, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Sections (1-μm) were cut and stained with 1% methylene blue for light microscopic examination. Gold or silver sections, stained with 2% uranyl acetate and 0.3% lead citrate, were examined with a Hitachi HS9 electron microscope.

Sporozoite transport in immune and naive chickens

Sporozoite transport within the intestinal mucosa and to extraintestinal sites was compared in naive and immune chickens.

Sporozoites within the intestinal mucosa: Five immune and 5 naive chickens of the same age were inoculated with 10×10^6 oocysts each, 17–18 days after the primary inoculation of the immune birds. At 3, 6, 12, 24, and 48 hr PI, 1 immune bird and 1 naive bird were killed by cervical dislocation. Sections of gut from the small intestine, liver, and spleen were removed and processed for light and electron microscopy as described above.

Immunoperoxidase staining utilizing rabbit anti-sporozoite sera diluted 1:500 with PBS was employed to stain paraffin sections as described earlier. The number of parasites in each of 3 compartments within the mucosa, i.e., the villous epithelium, lamina propria, and crypts was determined from counts of 50 villous-crypt units from each of the 3 areas of intestine described earlier. Only villous-crypt units that were sectioned longitudinally so as to include the crypt and the entire length of the villus were counted. The average number of parasites from the 3 areas was calculated for each chicken. Sections of liver and spleen were also examined for the presence of parasites. This experiment was replicated 3 times and the average number of parasites in each compartment of the gut (for each time period) was determined and expressed both as percentages of the total parasites in the mucosa and as total numbers.

Statistical analysis: The effect of immunity on the distribution of sporozoites in each compartment of the mucosa over time was determined using a split-plot analysis of variance. Counts of sporozoites in the villous epithelium, lamina propria, and crypts were transformed to common logarithms before analysis in an attempt to stabilize variance. The analysis of data was performed using the ANOVA procedure from the SAS system (SAS Institute Inc., Cary, North Carolina, available in the computer laboratory at the Ontario Veterinary College). The TEST statement was used where h = immunity|time and e = the error term, which in this case was trial·immunity·time.

Extraintestinal transport of sporozoites: The presence and viability of extraintestinal sporozoites in immune and naive donors infected with *E. maxima* was determined by the ability of their tissues to produce patent infections when fed to coccidia-free recipients. These experiments were based on the methods used by Long and Millard (1979). Five immune (17–18 days after immunization) and 5 naive birds of the same age were inoculated with 10×10^6 oocysts and killed at 3, 6, 12, 24, and 48 hr PI. Prior to opening the intestinal tract, the spleen and liver were removed to avoid contamination from the gut. Their livers, spleens, and intestinal mucosa were transferred to susceptible 5-wk-old birds, 1 organ per recipient. Recipient chickens were starved for 2 hr and were each fed 2 ml of an antacid mixture (8.0 g calcium carbonate, 3.4 g magnesium trisilicate, and 8.6 g colloidal kaolin in 30 ml water; Horton-Smith and Long, 1956), 10 min prior to the tissue transfers. Following the transfers, the recipients were individually housed in boxes in an isolation room. Feces were collected from day 5 to day 10 PT and total daily oocyst production was measured. If samples were negative, they were further examined by centrifugal flotation, which is a more sensitive detection method. A control recipient was included for each time interval to ensure the absence of extraneous infection. This experiment was replicated 3 times.

RESULTS

Identification of intraepithelial lymphocytes

Intraepithelial cells in the chicken small intestine were examined both at the light microscopic and at the electron microscopic levels. At the light microscopic level there were numerous intraepithelial mononuclear cells containing many large eosinophilic granules. There were 3 types of cells in addition to the epithelial cells. The first of these were goblet cells interspersed between the epithelial cells and extending to the level of the microvilli. The second and third groups of cells were similar to the intraepithelial lymphocytes (IEL's) described by Bjerregaard (1975).

Sporozoite transport in immune and naive chickens

Location of sporozoites in the intestinal mucosa: The immunoperoxidase staining of sporozoites in tissue sections enabled sections to be scanned at low power (10×) in order to observe patterns of sporozoite transport. At 3 hr PI, in both immune and naive birds, the majority of parasites appeared to be in the villous epithe-

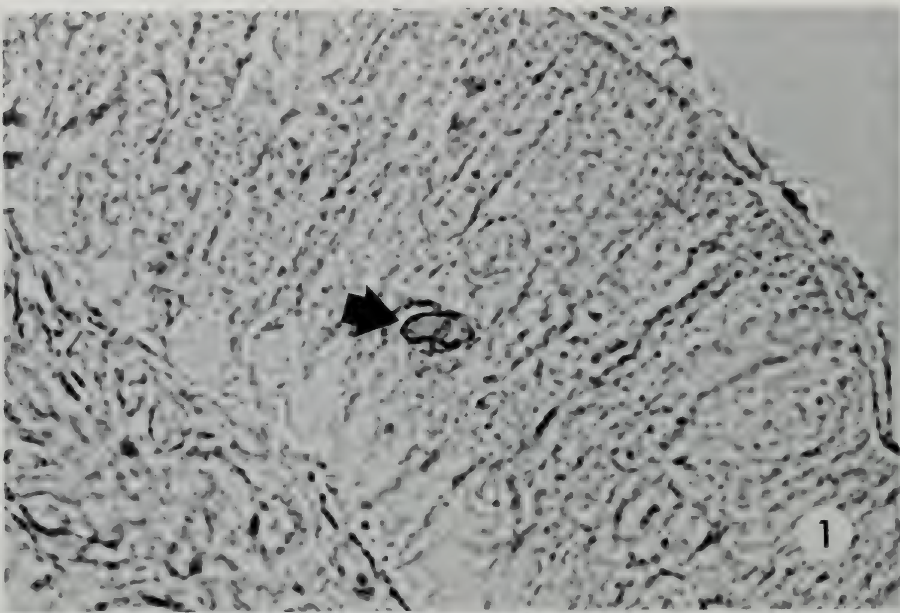


FIGURE 1. Sporozoite stained with immunoperoxidase stain, in villus of naive chicken at 3 hr postinfection, ×520. Arrow indicates parasitophorus vacuole.

lium, with a few in the lamina propria (Fig. 1). At 6 hr PI, there was still no apparent difference between naive and immune birds and the sporozoites were found in both the villous epithelium and the lamina propria. By 24 hr PI, an occasional sporozoite could be found in the crypts in naive birds. By 48 hr PI there was a marked difference between the immune and naive birds. In naive birds there were large numbers of parasites in the crypts, beginning to undergo development into schizonts. In immune birds, there were few, if any, parasites in the crypts and adjacent areas, but sporozoites persisted in the lamina propria.

The numbers of sporozoites in the villous epithelium, the lamina propria, and the crypts and adjacent area were determined for different time periods PI in both immune and naive chickens. The numbers of parasites in the different mucosal compartments were expressed as percentages of the total parasites in the mucosa (Fig. 2).

Figure 2 demonstrates several trends in the migration of sporozoites. At 3 hr PI, in the naive chicken the highest percentage of parasites was in the villous epithelium. Over the next 48 hr that proportion dropped to less than 30%. As the percentage of parasites in the villous epithelium dropped, the percentage in the lamina propria increased to over 50% at 24 hr PI. By 48 hr PI the percentage in the lamina propria had returned to approximately 25%. The percentage in the crypts was very low until 12 hr PI, after which point it increased slowly to reach 50% by 48 hr PI.

In immune birds, the percentages of sporozoites in the various compartments were similar to naive birds at 3, 6, and 12 hr PI. At 24 hr PI, the percentage of sporozoites in the villous epithelium was less than 10% and it remained this way at 48 hr PI. The percentage of parasites in the lamina propria at 24 hr PI was very similar to the percentage in naive birds, but unlike the naive birds, the percentage in this compartment at 48 hr PI was still high (approximately 60%). Finally, the percentage in the crypts increased slightly to 25% at 24 hr PI and was still at that level at 48 hr PI.

Analysis of variance indicated that there was a significant difference ($f = 3.63$, $P < 0.0029$) in the patterns of sporozoite distribution between naive and immune birds. The majority of the variation could be attributed to the differences between the immune and naive chickens from 24 to 48 hr PI ($f = 15.40$, $P < 0.0001$). Once this source of variation was removed there was no significant difference at the 1% level.

Sections of intestine processed for electron microscopy were examined in order to determine (1) the cell type that the sporozoite was transported in and (2) if there was any morphological evidence of damage to the sporozoites in immune birds. Sporozoites were observed in villous and crypt epithelial cells and in the 2 types of lymphocytes previously described, both within the epithelium and in the lamina propria (Figs. 3, 4). Sporozoites were found in both granulated IEL's and nongranulated IEL's, although they were not seen in IEL's with very large granules. Figure 4 shows a parasite in a nongranulated IEL. Sporozoites were not found free within the lamina propria.

There were no morphological differences observed between sporozoites in immune and naive birds at any time. The only difference noted was at 48 hr PI when developing schizonts were seen in naive birds but not in immune birds.

Extraintestinal transport of sporozoites: Transfers of tissues from infected donors to naive recipient birds were used to determine if viable parasites were present in extraintestinal sites. The development of a patent infection with a normal prepatent period in the recipient was considered evidence that viable sporozoites had been present in the tissue transferred. Transfers of intestinal mucosa were used as controls since sporozoites had already been demonstrated in the mucosa up to 48 hr PI at the light and electron microscopic level. All transfers of intestinal mucosa were successful and the resulting total oocyst production ranged from 1×10^5 to 1×10^8.

Transfers of liver did not yield consistent results but at least 2 of the 3 attempts were successful with both immune and naive donors at each time interval, up to 48 hr PI. Total oocyst production ranged from 5×10^4 to 5×10^7 oocysts. There was no apparent difference between immune and naive donors; 13 out of 17 transfers were successful with naive donors while 14 out of 17 were successful with immune donors.

Spleen transfers were the least successful; 11 out of 17 were successful from naive donors and 10 out of 17 were successful from immune donors. The time of transfer was not useful for predicting the success of these transfers, at least 1 transfer was successful at each time period with the exception of 12 hr PI with an immune donor. However the liver and gut of this donor were used successfully for transfers.

The pattern of oocyst production by the recipients was also noted. When transfers were made at 3, 6, and 12 hr PI, peak oocyst production occurred on days 7 and 8 PT (equivalent to day 7 or 8 PI of the donors) regardless of the source of the transfer. In a few cases, where overall numbers of oocysts were very low, there was no "peak" day of production and oocysts were observed from days 7 to 9 PI. When transfers were given 24 hr or 48 hr PI the oocyst production of the recipients peaked at day 7 or 8 PT (days 8–10 PI).

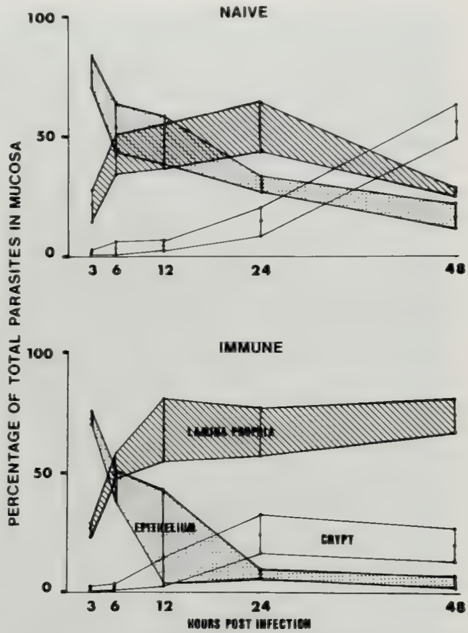
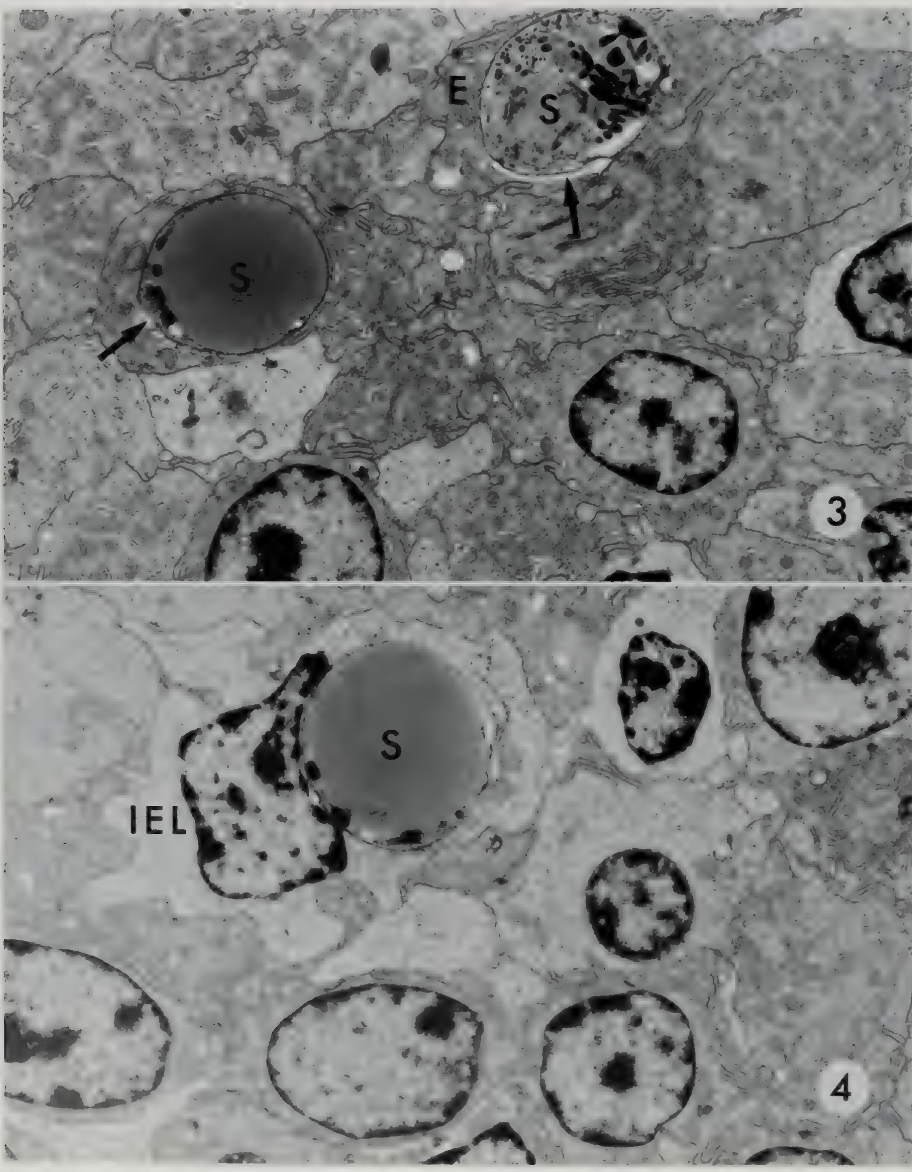


FIGURE 2. Comparison of the distribution of *Eimeria maxima* in the intestine of naive and immune chickens at 3, 6, 12, 24, and 48 hr PI. Percentage of total parasites in mucosa is the mean percentage of total parasites ± 1 standard deviation in each compartment for each time period.

DISCUSSION

The transport of sporozoites by IEL's in the intestinal mucosa differs significantly between immune and naive chickens. No differences, however, were noted in the extraintestinal transport of sporozoites in immune and naive birds. These results suggest some intricate relationships between the sporozoites, the IEL's, and the host epithelial cells.



FIGURES 3, 4. Electron micrographs of intracellular *Eimeria maxima* sporozoites 3 hr postinfection, × 7,500. **3.** Sporozoites (S) in villous epithelial cells (E). Arrows indicate parasitophorus vacuoles. **4.** Sporozoites (S) in intraepithelial lymphocyte (IEL) in villous epithelium.

Characteristics of the transport cell

Our observations on the ultrastructure of the intraepithelial cells in chickens were comparable to those of Bjerregaard (1975), and his terminology of intraepithelial lymphocytes (IEL's) and granulated IEL's (gIEL's) are used in this paper, with the understanding that this classification is based purely on morphology and does not denote any function. The clarification of the function of these cells requires the same type of work that is already well underway in rodent and human sys-

tems, including the development of monoclonal antibodies to label the subsets, the separation of IEL's and gIEL's into various subsets, and functional assays on the individual subsets (Ernst et al., 1985a; Dobbins, 1986). Only then will we be able to assess which cells transport sporozoites.

Sporozoites were observed in both IEL's and gIEL's although they were never observed in gIEL's with large granules. This may reflect a preference for a particular subset characterized by small granules, or more likely, may be due to a limiting factor of size. Ernst et al. (1985b) have already shown that the presence or absence of granules does not correlate with functional differences.

The morphology of the sporozoites in immune and naive chickens 48 hr PI was identical and the results of the transfers of gut mucosa indicated that they were still viable. This suggests that either the IEL is not an integral part of the immune response to coccidiosis, or that the sporozoite has developed mechanisms to escape the detrimental effects of the IEL.

Distribution of parasites

The pattern of distribution of sporozoites among the compartments in the immune birds between 24 and 48 hr PI was significantly different from that in the naive birds. Significantly fewer sporozoites arrived at the crypt in immune birds implying there is some inhibition of transport of lymphocytes from the lamina propria to the crypts in immune birds.

These findings are in contrast to the results of Rose et al. (1984), which indicated that the transport of sporozoites of *Eimeria tenella* from the surface epithelium to the crypts was not inhibited in immune birds. However, these authors found an inhibition of the transfer of sporozoites from the IEL into the crypt epithelial cell. They also found an overall reduction in the numbers of parasites in immune chickens as compared to naive birds. This trend was also seen in our experiments but was not statistically significant. Mesfin and Bellamy (1979) also found that sporozoites of *E. falciformis* migrated equally well in naive and immune mice.

These inconsistencies may have been due to differences in the species examined; *E. tenella* develops in the cecum rather than the small intestine of the chicken and is far less immunogenic than *E. maxima*. Another reason for the discrepancy may have been the fact that earlier experiments were done using histochemical sporozoite stains. There is a marked infiltration of inflammatory cells into the lamina propria in immune chickens (Rose, 1978), which may obscure histochemically stained sporozoites and result in erroneous results. The immunoperoxidase stain is less likely to be affected by an influx of cells since the stain did not stain host cells.

Sporozoite migration to the crypt was not completely inhibited, however, and since *E. maxima* induces complete immunity, as judged by oocyst production, it is unlikely to be the only factor involved. The prevention of development of first-generation schizonts is likely the most important factor since there appears to be total inhibition at that point. Occasional schizonts were seen within crypt epithelial cells but they were usually abnormal in appearance. Whether the inhibition of schizogony is due to prevention of sporozoites from entering the epithelial cell, previous damage to the sporozoite or inhibition once within the epithelial cell is unknown.

The presence of viable sporozoites in the peripheral circulation of both naive and immune chickens PI, as shown by the transfer experiments, suggests that transport to extraintestinal sites is a reflection of normal lymphocyte traffic.

Extraintestinal transport of sporozoites

Infections were induced in susceptible chickens by the transfer of tissues from either naive or immune chickens, up to 48 hr PI. This demonstrated that stages of *E. maxima* were capable of surviving in extraintestinal tissues up to 48 hr PI. No differences were found between the infections induced by transfers of "immune" mucosa or those induced by transfers of "naive" mucosa at any of the times PI.

Peak oocyst production in recipients of liver and spleen tended to occur on days 7 or 8 PI. This is the same pattern one sees in natural infections which implies that the transferred stage was the sporozoite. The survival of the sporozoite in immune birds was remarkable considering that serum antibodies have a deleterious effect on sporozoites. The most likely explanation for their survival is that they do not leave their transport cells while in the peripheral circulation.

Histological examination of liver and spleen stained with the sporozoite stain did not reveal any sporozoites. There are several possible reasons for this. Only small numbers of sporozoites are required to produce patent infections in recipient birds and these would be difficult to de-

tect in histological sections. Also, sporozoites frequently leave cells as soon as the microenvironment becomes unsuitable. Fernando (pers. comm.) has seen sporozoites leave mononuclear cells that were obtained after density gradient centrifugation of spleen cells. There is therefore a possibility that the sporozoites leave cells during fixation and are destroyed.

In summary, we have shown that transport of sporozoites within the intestinal mucosa of immune chickens differs significantly from transport in naive birds. Fewer IEL's carrying sporozoites are found at the crypts in immune chickens by 48 hr PI. Since we do not understand the normal migration pattern of IEL's, either within the mucosa or extraintestinally, it is not possible to determine whether IEL's are inhibited in immune chickens or directed to migrate to the crypts in naive chickens. However, the inability of IEL's carrying sporozoites to migrate to the crypts in immune chickens implies that the parasite has some control over these cells.

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IN VITRO CULTIVATION OF A BABESIA ISOLATED FROM A WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

Patricia J. Holman, Kenneth A. Waldrup, and Gerald G. Wagner

Texas Agricultural Experiment Station, Department of Veterinary Microbiology and Parasitology, Texas A&M University, College Station, Texas 77843

ABSTRACT: Pyriforms and ring forms of *Babesia odocoilei* were detected in thin blood smears obtained from a white-tailed deer killed by a hunter in Anderson County, Texas. Erythrocytes from the deer were cultured and the parasites maintained through 8 serial subcultures during 1 mo. The parasite was successfully established in culture using Medium 199 supplemented with either 20% deer serum or 40% normal adult bovine serum. The highest parasitemia observed was 30% and more than 4 parasites per erythrocyte were often observed. Cultured *B. odocoilei* remained infective for a susceptible white-tailed deer.

The babesia of white-tailed deer (*Odocoileus virginianus*) in the southern U.S.A. (Spindler et al., 1958; Emerson and Wright, 1968; Perry et al., 1985) is known as *Babesia odocoilei* (Emerson and Wright, 1970). The epidemiological significance of this hemoprotozoan is currently being evaluated (Waldrup and Wagner, 1986). Since natural infections in deer are characterized by extremely low numbers of circulating parasites, an *in vitro* method of obtaining quantities of the parasite would be advantageous in this research.

The microaerophilous method of *in vitro* propagation of *Babesia bovis* (Levy and Ristic, 1980) has provided a convenient means of establishing pure cultures and maintaining stocks of *Babesia* spp. Adaptation of this method and the successful cultivation of *B. odocoilei* isolated from white-tailed deer are described.

MATERIALS AND METHODS

Babesia odocoilei

As part of an ongoing study of deer diseases, 8 ml deer blood infected with *B. odocoilei* was collected in sodium citrate (1.2% final concentration) by cardiac puncture. The blood was kept on ice for 3 days and then refrigerated upon arrival in the laboratory. The deer, designated E-1, was a 1½-yr-old white-tailed male (*O. virginianus*) killed by a hunter at the Gus Engeling Wildlife Management Area in Anderson County, Texas (96°26′W, 32°55′N).

Donor erythrocytes

Deer blood was obtained from a 5-mo-old *Babesia* spp.-free white-tailed female by jugular venipuncture. Erythrocytes (rbc's) for culture were obtained by defibrinating whole blood and then centrifuging at 1,000 *g* for 20 min. The serum was decanted and stored at −70 C for later use. The buffy coat was removed and discarded, and an equal volume of Puck's saline glucose (GIBCO Laboratories, Grand Island, New York) plus extra glucose (PSGG; see below) was added to the rbc pellet. The cells were resuspended and stored at 4 C.

Media

Growth medium (M-199 with Earle's salts, without L-glutamine; KC Biological, Lenexa, Kansas) was supplemented with 20% deer serum (female donors) or 40% bovine serum (adult steer donor), 20 mM TES (N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; 2-(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl) aminoethanesulfonic acid; Sigma, St. Louis, Missouri), 2 mM L-glutamine (KC Biological, Lenexa, Kansas), and 200 μg/ml garamycin (Schering Corporation, Kenilworth, New Jersey). PSGG was prepared by adding 20 g glucose/L of Puck's saline glucose plus 10 ml antibiotic–antimycotic solution (final concentration of 100 U penicillin, 100 μg streptomycin, and 25 μg fungizone/ml; GIBCO, Grand Island, New York).

Culture

Babesia odocoilei cultures were initiated 5 days after the collection of infected blood. Unwashed infected rbc's were removed by pipet from the bottom of the sedimented cell layer and 0.1 ml added to 1.15 ml growth medium in each of 2 wells of a 24-well culture plate incubated at 37 C in a humidified 5% CO_2 in air atmosphere. The cultures were fed daily by removing 1 ml supernatant and adding 1 ml fresh growth medium. Giemsa-stained thin smears were made periodically from cultured rbc's to monitor parasite multiplication. The first 2 subcultures were performed by feeding the culture as described; the cultured rbc's were then resuspended and 0.25 ml transferred to another well containing 0.9 ml growth medium and 40 μl washed E-1 rbc's. Quantities for subsequent subcultures were 0.25 ml culture, 0.9 ml growth medium, and 0.1 ml donor deer rbc suspension.

Deer and bovine serum comparison

Ten days after collection, stored E-1 blood was centrifuged at 1,000 *g* for 20 min. The plasma and buffy coat were removed and discarded. The rbc pellet (approximately 3 ml) was resuspended in 20 ml M-199 and washed by centrifugation 3 times, each time removing the buffy coat. After the final wash, the remaining 2 ml packed rbc's were resuspended in 2 ml

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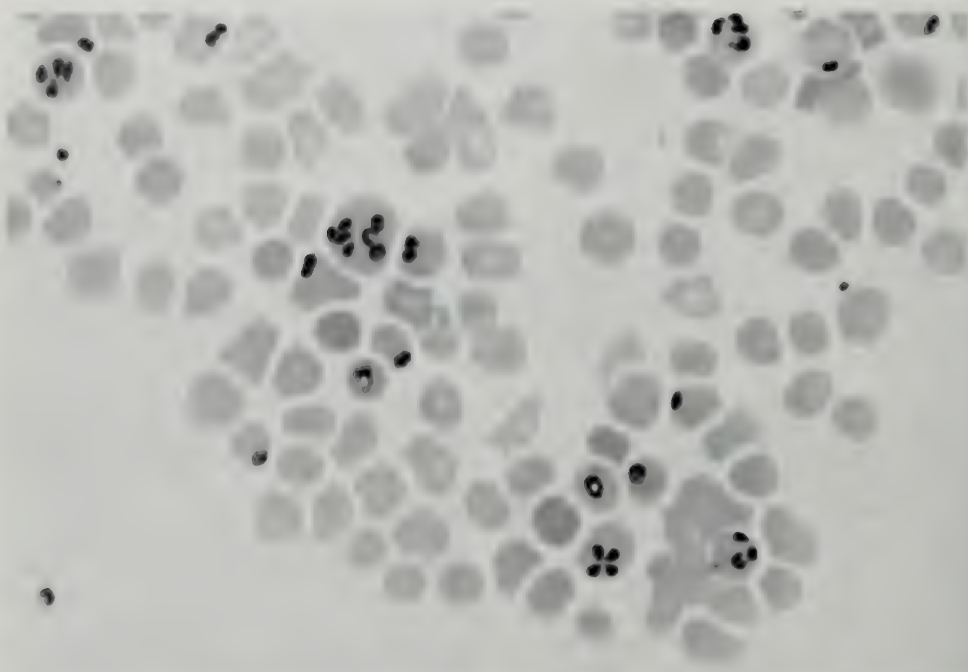

FIGURE 1. Typical culture forms of *B. odocoilei* showing round and ring forms, pyriforms, peripheral forms, and multiply-infected cells.

PSGG. Quadruplicate cultures were initiated using 0.15 ml E-1 washed cell suspension and 1.1 ml growth medium containing either 20% deer serum or 40% adult bovine serum. The cultures were fed daily with medium containing the appropriate serum and monitored by Giemsa-stained thin smears.

Cryopreservation

Cultures of *B. odocoilei* were cryopreserved by methods similar to those described for *B. bovis* (Palmer et al., 1982). The freezing medium was PSGG with 10% PVP (polyvinylpyrollidone-40; Sigma, St. Louis, Missouri) as the cryoprotectant. Briefly, cultures with a parasitemia greater than 10% were centrifuged for 10 min at 1,000 *g*. The supernatant was discarded, the rbc pellet resuspended with an equal volume of cold freezing medium, and 0.2 ml was placed in each cryopreservation vial. The vials were submerged in an ethanol bath at −50 C overnight and transferred to a liquid nitrogen storage tank 24 hr later. To resuscitate, the vial contents were thawed rapidly in a 37 C waterbath and transferred to a well of a 24-well culture plate. The volume was brought to 1.25 ml by the addition of 0.1 ml fresh deer rbc's and 0.95 ml growth medium, and the plate incubated at 37 C in a humidified 5% CO_2 in air atmosphere. The cultures were fed 1 ml fresh medium daily.

Cultured *B. odocoilei* infectivity

A 5-mo-old *Babesia* spp.-free white-tailed female was inoculated intravenously with 1.58×10^9 infected rbc's in the eighth subculture. Beginning 4 days after inoculation, blood samples were obtained and thin smears examined every 2–3 days for 3 wk. The packed cell volume (PCV) was determined every 2 days for 10 days, then on days 15 and 22.

RESULTS

Parasites in deer blood

Microscopic examination of Giemsa-stained thin blood smears from E-1 showed a mixed infection of theileria and *B. odocoilei*. The babesia occurred as single, paired, or multiple pyriforms or single, paired, or multiple "ring" forms. There were too few babesia-parasitized cells to determine a percent parasitemia (typically 1 infected rbc/6,000 or more rbc's examined).

The rbc donor deer was also infected with theileria, with a consistent parasitemia of 2.5–3% throughout the several months the deer was used. However, this parasite did not proliferate under the culture conditions used; therefore this deer served as the donor for fresh rbc's.

Babesia odocoilei culture

At the time of initiation the percent parasitemia was too low to determine accurately, al-

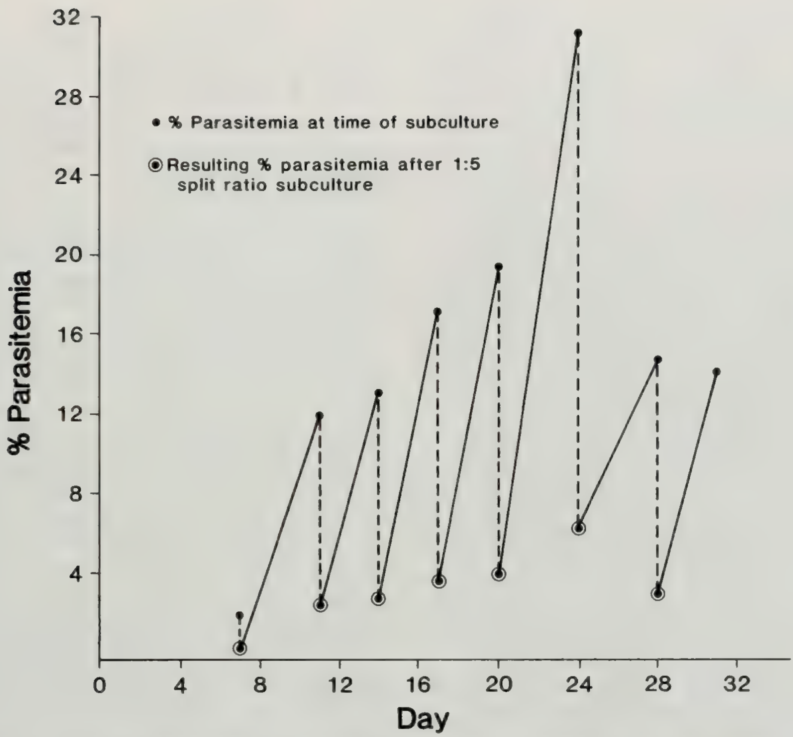

FIGURE 2. Consecutive subcultures of *B. odocoilei* from the time of culture initiation from infected E-1 blood through the eighth passage *in vitro*.

though a few parasites could be found on thin smears (typically 1 infected rbc/6,000 or more rbc's examined). On day 7 the first subculture was performed. The parasitemia was 1.9% and was comprised of single ring forms and large and small pyriforms, paired round forms and pyriforms, and 4 or more pyriforms within a single erythrocyte; pyriforms were frequently observed on the periphery within the cell (typical forms are shown in Fig. 1). By day 11, the parasitemia reached 11.9% and subculture 2 was performed. The next 2 subcultures were each done at 3-day intervals at 17.6% and 19.4% parasitemia, respectively. Subculture 5 was delayed until 4 days later to determine if the parasites would continue to multiply after 3 days. The parasitemia reached 31.1% with many rbc's containing more than 4 and as many as 12 parasites per cell. After this subculture, multiplication slowed so that 4 days later the parasitemia was only 14.7%. The culture was subcultured and 3 days later the parasitemia was again 14.1%. The overall pattern of multiplication and subculture is shown in Figure 2.

Comparison of deer and bovine serum-supplemented media

Giemsa-stained blood smears of 10-day-old E-1 cells after washing contained very few parasites and these appeared enlarged compared to those seen in the original E-1 smear. When cultured, the parasitemia remained low until day 8, at which time it rose to 2.2% in deer serum and 3.4% in bovine serum cultures. The multiplication of *B. odocoilei* in the 2 media is given in Table I.

Cryopreservation

The babesia culture was successfully cryopreserved. Upon resuscitation, the parasite recovered well, achieving a 17.3% parasitemia 4 days after thawing as shown in Figure 3.

Cultured *B. odocoilei* infectivity

The presence of *B. odocoilei* in thin blood smears was first detected 4 days after inoculation of the deer and persisted at low levels throughout the 3-wk observation period (Table II). The PCV

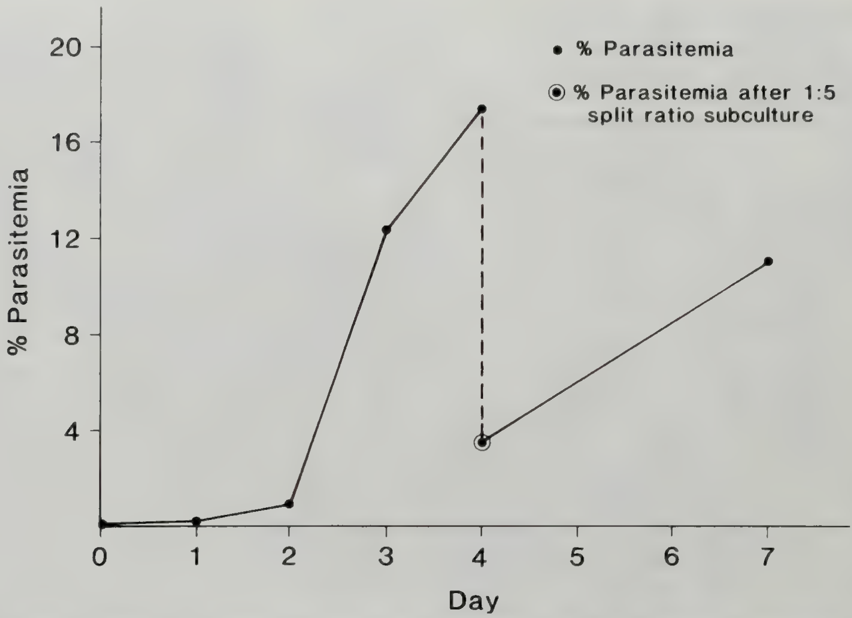

FIGURE 3. Growth of *B. odocoilei* in culture upon resuscitation from liquid nitrogen frozen storage.

declined from 53% on day 4 after inoculation to 30% on day 10 (Table II). The PCV returned to a normal level (50%) by day 15.

DISCUSSION

The *B. odocoilei* isolated from a white-tailed deer adapted readily to *in vitro* cultivation. Although the number of parasites in the deer blood sample was extremely low at culture initiation, the parasites multiplied rapidly and continued to thrive. This was partly due to the high suitability of previously reported culture parameters (Levy and Ristic, 1980) for this parasite, and partly due to the fact that *B. odocoilei* propagates easily *in vitro* in comparison with some other babesias. The ease of propagation is clearly demonstrated by the successful cryopreservation of this parasite. Although *B. bovis* (Levy and Ristic, 1980), *B. bigemina* (Vega et al., 1985a), *B. divergens* (Konrad et al., 1984; Pudney, 1984), *B. major* (Donnelly et al., 1984), and *B. canis* (Moreau and Laurent, 1984) have been continuously cultured by similar methods, only *B. bovis* (Palmer et al., 1982) and *B. bigemina* (Vega et al., 1985b) cultures have been initiated using cryopreserved

TABLE I. *Comparison of deer babesia initiation and growth in deer serum (20%)- or bovine serum (40%)-supplemented medium.**

Day	Deer serum culture	Bovine serum culture	Bovine serum subculture
0	<0.1	0.1	
4	<0.1	<0.1	
6	>0.1	>0.1	
8	2.2	3.4	
9	3.9	5.4	(1.0)†
12	11.4	4.1	4.8

* All values are percent parasitemia

† Estimated percent parasitemia foll[illegible]ure of a bovine serum-supplemented culture.

TABLE II. *Parasitemias and packed cell volumes from a deer infected with cultured* B. odocoilei.

Day	Observed *B. odocoilei*	Packed cell volume
0	NPS*	ND†
4	1/5,000	53
6	1/10,000	43
8	1/8,900	37
10	1/12,000	30
13	1/30,000	ND
15	NPS	50
17	1/15,000	ND
20	1/15,000	ND
22	1/11,000	52

* No parasites seen.

† Not done.

cultures. In addition, *B. odocoilei* does not appear to be sensitive to white blood cells in culture as reported for other *Babesia* spp. (Timms, 1980), nor is centrifugation deleterious as with *B. bovis* (Levy and Ristic, 1980).

A high concentration of garamycin was used in the *B. odocoilei* cultures due to the manner of blood collection and the resulting questionable sterility; this was not inhibitory to the babesia. Penicillin and streptomycin have been reported in the establishment of *B. bovis* cultures (Levy and Ristic, 1980), and we have used penicillin, streptomycin, and fungizone in combination with no ill effects to cultured *B. bovis, B. bigemina,* or *B. odocoilei.*

Cultured *B. odocoilei* were able to utilize bovine serum which is more readily available than deer serum. Related studies of *B. bovis* and *B. bigemina* cultures in this laboratory have required 40% bovine serum and this amount was needed for successful cultures of *B. odocoilei.* However, because of the difficulties in obtaining sufficient deer serum, we were limited to 20% supplementation, but with good results.

The ease with which the babesia of white-tailed deer was cultured and cryopreserved provides an opportunity for simple maintenance of the parasite in the laboratory for further study, avoiding difficulties associated with using the natural host as a source of material.

ACKNOWLEDGMENTS

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TEGUMENTAL GLUCOSE PERMEABILITY IN MALE AND FEMALE *SCHISTOSOMA MANSONI*

E. M. Cornford, A. M. Fitzpatrick, T. L. Quirk, C. P. Diep, and E. M. Landaw*

Comprehensive Epilepsy Program, Research and Neurology Services, V. A. Wadsworth Medical Center, Los Angeles, California 90073, and
Department of Neurology, UCLA School of Medicine, Los Angeles, California 90024-1769

ABSTRACT: Tegumental hexose transporters have been kinetically characterized in mated and separated male and female *Schistosoma mansoni* 8–12 wk postinfection. Significant gender-specific differences in K_m and V_{max} were observed. In mated males, the estimated constants (mean ± SE) were: $K_m = 0.63 \pm 0.31$ mM, $V_{max} = 0.93 \pm 0.44$ nmol/mg worm water/min, and the $K_d = 0.25 \pm 0.09$ μl/mg worm water/min. In mated females the kinetics were: $K_m = 0.99 \pm 0.40$ mM, $V_{max} = 1.22 \pm 0.42$ nmol/mg worm water/min, and $K_d = 0.60 \pm 0.14$ μl/mg worm water/min. The influx of 2-deoxy-D-glucose and 3-O-methylglucose has been similarly characterized; these analogs share the same glucose transporter in male and female schistosomes. 2-Deoxy-D-glucose has a higher affinity, and 3-O-methylglucose a lower affinity, than does glucose. Because mated male schistosomes supply glucose to female partners, similarities between the free glucose concentration of the male and the affinity of the transporter determined for mated female schistosomes suggest that male-to-female transfer may be a potentially rate-limiting step in glucose utilization by the female. Permeability × surface area (PS) products and V_{max}/K_m ratios were significantly elevated in mated schistosomes, suggesting that the transporter is primarily localized to the dorsal surface of the male. Gender- and mating-specific analyses of PS products indicate that tegumental permeability to glucose is significantly increased in mated schistosomes, and compares very favorably to that of the host liver.

The schistosome tegument has certain features that contribute to survival of the parasite within its vertebrate host (Podesta, 1982). Despite the presence of a functional gut, the external surfaces of schistosomes have been recognized as an additional site for assimilation of metabolites (Fripp, 1967; Senft, 1968; Rogers and Bueding, 1975). Transport mechanisms, which impart selective permeability of metabolites to the schistosome integument, have been defined for several classes of compounds. Carrier systems for the transport of hexoses (Isseroff et al., 1972; Uglem and Read, 1975; Podesta and Dean, 1982b), amino acids (Chappell, 1974; Asch and Read 1975a, 1975b; Isseroff et al., 1976; Mercer and Chappell, 1985), purine and pyrimidine compounds (Levy and Read, 1975), and choline (Young and Podesta, 1985) have been described in *Schistosoma mansoni.* Others have examined the male and female surfaces for gender-specific functions. No difference in incorporation between males and females was seen for glycine (Asch and Read, 1975b) or choline (Young and Podesta, 1984, 1985). Cornford et al. (1981) could not find significant differences in glucose uptake kinetics when separated male and female *Schistosomatium douthitti* from murine infections were compared.

Coles (1973) suggested a need for the comparative study of mated and unmated schistosomes, and of male and female worms, to provide insight into schistosome metabolism and further understanding of sex-specific drug actions. The copulative state of schistosomes is now known to impart dramatic differences in tegumental influx of nucleic acid precursors (Pica Mattoccia et al., 1982) and acidic amino acids (Cornford, 1985). Other evidence has suggested that hexose uptake may differ in males and females, and influx was greater in mated than unmated schistosomes (Cornford and Huot, 1981; Cornford, 1982). In contrast, Mercer and Chappell (1986) report that glucose uptake rate was higher for separated males and females than for equivalent worm pairs. Because it has been established that hexoses are transferred from (mated) male to female schistosomes (Cornford and Huot, 1981) in a very short time (i.e., less than 1 min), short-duration (5-sec) isotopic pulse-labeling might represent an optimal method for defining transport kinetics and comparing mated and unmated flukes. Furthermore, incubation times of less than 1 min are widely considered necessary to measure transporter-limited influx (Kashiwagi et al., 1983; Whitesell and Abumrad, 1985; Lanks, 1986).

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* Department of Biomathematics, UCLA School of Medicine, Los Angeles, California 90024-1766.

In the present study, we used internal reference isotope methods (Cornford, 1982) for the study of carrier-mediated tegumental transport of glucose, 2-deoxyglucose (2-DG), and 3-O-methylglucose (3-O-MG) in male and female *S. mansoni*. In addition, comparisons were made between mated and unmated schistosomes. The kinetic parameters of transport (maximal velocity, half-saturation constant, and diffusion component) were defined from a nonlinear regression analysis of uptake velocities as a function of substrate concentrations.

MATERIALS AND METHODS

Schistosomes (a Puerto Rican strain of *S. mansoni*) were perfused from the veins of male or female Swiss-Webster mice by the method of Duvall and Dewitt (1967). They were washed in RPMI-1640 (GIBCO, Santa Clara, California) medium and maintained *in vitro* at 38 C. Fresh RPMI-1640 culture medium was infused every 20–30 min (50–75% of the total volume being withdrawn and replaced), and schistosomes were not maintained *in vitro* for longer than 1 hr. All influx studies were initiated within 30 min after the hosts had been humanely killed. Schistosomes were rinsed once in schistosome saline containing the same concentration of glucose as the test isotopic medium prior to immersion in radioactive media to ensure that the specific activity of the [^{14}C] glucose was not altered by passively transferred substrate.

Radioisotopes

Isotopes were purchased from New England Nuclear Corporation (Boston, Massachusetts). Specific activities (in mCi/mmol) of the ^{14}C metabolites were: [U-^{14}C] D-glucose 348, [1-^{14}C] D-glucose 55, [2-^{14}C] D-glucose 57, [3,4-^{14}C] D-glucose 10, [6-^{14}C] D-glucose 57, [U-^{14}C] 3-O-methylglucose 329, [methyl-^{14}C] 3-O-methylglucose 55, [U-^{14}C] 2-deoxyglucose 282, and [^{3}H] water 1 mCi/g. The ^{113m}In generator was purchased from NEN Radiopharmaceuticals (North Billerica, Massachusetts). To each 1.0 ml of indium eluted, 10 μl of sterile disodium EDTA solution (150 mg/ml; Endrate, Abbott Laboratories, North Chicago, Illinois) was added. The pH of the solution was adjusted to about 7.0 by the dropwise addition of sterile 0.1 N NaOH (about 40 μl), and the pH fixed at 7.55 by adding 100 mM HEPES (N-2-hydroxyethyl piperazine N-2-sulfonic acid; obtained from Calbiochem, La Jolla, California) to give a final buffer concentration of 10 mM.

Incubations

Incubations in isotopic media were performed at 38 C in a modified schistosome saline (Bueding, 1950; Cornford et al., 1981) containing 10 mM HEPES buffer. The incubation mixture (usually a total volume of 1.5 ml) typically contained about 1.5–2.0 μCi of ^{14}C-labeled test metabolite (hexose), a 10-fold greater concentration (10–20 μCi) of tritiated water, and 0.1–0.2 mCi of [^{113m}In] EDTA. Over the range of glucose concentrations tested, 0.002–50 mM, alterations in the specific activity of glucose were achieved by using a constant quantity (about 2.0 μCi) of [^{14}C] hexose and final concentrations adjusted by the addition of unlabeled glucose.

Groups of 4 or 6 pairs of schistosomes (either all mated, or all separated) were placed in containers with a nylon mesh bottom to permit rapid transfer from culture media to isotopic solution. (Either 1 container of 6 pairs of worms or 2 containers of 4 pairs were tested, so that 6 or 8 pairs of schistosomes were exposed to each different glucose concentration.) Worms were removed from the RPMI-1640 culture medium, rinsed in buffered saline (or buffered saline containing glucose), and transferred into the buffered saline–isotope mixture. After a 5-sec exposure to isotopes, the schistosomes were removed, metabolism was arrested by flooding the worms with ice-cold silicone oil (Aldrich Chemical Company, Milwaukee, Wisconsin), and worms were prepared for scintillation counting (Cornford, 1985). Triplicate 10-μl samples of this mixture were subsequently transferred to liquid scintillation vials for determination of relative isotope content.

Liquid scintillation counting

As soon as the schistosome tissue had been digested, the pH of the solution was brought to near neutrality by the addition of approximately 35 μl of analytical grade (glacial) acetic acid (to minimize chemiluminescence), and 5 ml of scintillation fluid (Econofluor, Beckman Instruments) were added to each vial. The samples were assessed for indium content (1 min) in a Packard 460C (or a Beckman LS 5800) scintillation counter with the windows set as described by Oldendorf and Szabo (1976). Days later, after all the ^{113m}In ($T_{1/2}$ = 100 min) had decayed, the vials were recounted for tritium and ^{14}C content. The net indium counts were obtained by subtraction and appropriate decay correction as described previously (Cornford et al., 1978). When vials were sequentially assayed for indium disintegrations in 1 min, each successive vial had to be decay-corrected for the elapsed time (i.e., 1.0 min plus the time required by the machine to change samples). In the Packard 460C counter this interval is about 0.23 min, and varies as a function of the sample number. In the Beckman LS 5800, the microprocessor decay correction program automatically corrects for both isotope decay and time loss.

Tissue uptake indices

The schistosome integumental influx, or tissue uptake index (=TUI) of the various [^{14}C] glucoses was determined as described previously (Cornford et al., 1982). The TUI is a ratio of the distribution volumes (=DV) of the test metabolite ([^{14}C] glucose) and the 2 reference isotopes:

$$\mathrm{TUI} = 100\%\left(\frac{{}^{14}\mathrm{C\ DV} - {}^{113m}\mathrm{In\ DV}}{{}^{3}\mathrm{H\ DV}}\right)$$

where the distribution volume = (dpm/μl worm water)/(dpm/mg medium). In the TUI ratio, the minuend represents the total glucose radioactivity in the schistosome (both incorporated and passively carried on the external surface). The subtrahend (the distribution volume of ^{113m}In) estimates that fraction of test substance which is extrategumental, since the polar [^{113m}In]

EDTA chelate is excluded by intact membranes (in a manner similar to radioiodinated serum albumin). This technique measures tegumental uptake exclusive of the functions of the schistosomal digestive system (Cornford and Oldendorf, 1979). The distribution volume of [^{3}H] water is a function of schistosome mass, and permits expression of uptake relative to traditional parameters such as weight or protein content (Cornford et al., 1982). Other workers (Podesta and Dean, 1982a, 1982b) suggest that the use of [^{3}H] water to estimate mass may be subject to variations as a function of glucose concentration. To preclude this possible problem in our study, uptake was expressed as a function of the mean water distribution. Thus, parameters of schistosome mass have been normalized for the range of glucose concentrations employed.

Uptake velocities were derived from the TUI as described previously (Cornford et al., 1981), except that different relationships for the [^{3}H] water distribution volume (DV) and protein content have been established in mated and separated schistosomes. Studies of the distribution volume of [^{3}H] water (at equilibrium), in both mated and separated male and female schistosomes, indicated that slightly greater water distribution volumes were apparent in separated than in mated worms (55 days postinfection [PI]). Mated males have 0.253 ± 0.04 mg water (mean $\pm$ SD), as compared to 0.286 ± 0.03 mg water in separated males. Mated females contained 0.113 ± 0.02 mg water, and 0.132 ± 0.02 mg water was measured in separated females ($n = 13$ for each SD). Dried male worms perfused at the same age postinfection weighed an average of 0.110 mg, and females, 0.030 mg; values that were consistent with the data of Lennox and Schiller (1972). Thus, the worm water : dry weight ratios are 2.60 (separated) and 2.30 (mated) for males, and 4.40 (separated) and 3.77 (mated) for females. After a 5-sec exposure to tritiated water, the DV is 0.61 (61% saturated) in mated males, 0.36 maximum in mated females, 0.57 maximum in separated males and 0.45 maximum in separated females.

The [initial velocity/substrate concentration] was derived as follows: $V/S = [(^{14}C\ DV) - (In\ DV)] \times$ (mean ^{3}H DV)/R/12 min, where R = the ratio of the (^{3}H DV at 5 sec)/(^{3}H DV at equilibrium). Uptake velocities were thus expressed as nmoles/mg worm water/min. Methods for conversion of uptake index data into uptake velocities and the derivation of estimates of kinetic constants are described in detail elsewhere (Pardridge, 1983).

Analysis of data

The Michaelis–Menten plus diffusion relation between uptake velocity V (nmoles/mg worm water/min) and substrate concentration S (mM) is:

$$V = S \times \left(\frac{V_{max}}{K_m + S} + K_d\right).$$

V_{max} (same units as V) is the maximal influx velocity for the saturable component, K_m (mM) is the half-saturation constant, and K_d (μl/mg worm water/min) is the diffusion constant for the nonsaturable influx component. As the measured TUI is directly proportional to V/S and has a fairly constant measurement error variance over the range of substrate concentrations employed, $V_{max}/(K_m + S) + K_d$ was fit to the observed values of V/S by unweighted least squares. Program BMDP3R (Dixon, 1985) was used for each nonlinear regression analysis to obtain estimates and asymptotic standard errors for V_{max}, K_m, K_d, and the (unsaturated) permeability × surface area product, $PS = V_{max}/K_m + K_d$. In the analysis of unlabeled glucose (substrate) inhibition of labeled 3-O-methylglucose or 2-deoxy-D-glucose transport, the name of the half-saturation constant is changed to K_i and is the only parameter estimate reported.

For each experimental isolation of schistosomes from a host, 4 separate regression analyses were performed, each corresponding to 1 of the 4 combinations of sex (female vs. male) and mating status (*in copula* vs. separated). In order to test the significance of any sex effect or mating effect on a particular regression parameter, the estimates from the 4 regressions were compared among themselves and across different experiments by a repeated measures analysis of variance (program BMDP2V, Dixon, 1985). Because of the large, near constant coefficient of variations of the estimates of K_m and V_{max} and the skewness in their distributions, these particular parameter estimates were log-transformed prior to implementing the repeated measures analyses. Likewise, their summary statistics across experiments are expressed as geometric means (antilog of the mean of the logarithms) and not arithmetic means. The variance stabilizing transform for the K_d analysis was the square root; all other parameters were analyzed untransformed.

Disintegrations per minute (dpm) were derived by regression analyses and comparison of standard quench curves stored in the microprocessor unit of the scintillation counter.

RESULTS

Initial results confirmed previous work (Isseroff et al., 1972; Uglem and Read, 1975) indicating that glucose influx in *S. mansoni* occurs by a saturable, carrier-mediated process. Mating and gender-specific comparisons of glucose influx by schistosomes are graphically presented in Figure 1. Glucose influx (as measured by TUI's) did appear to be influenced by the mating status. Over a wide range of concentrations, mated males accumulated more glucose than did unmated males. Similarly, at any given glucose concentration, the mean glucose uptake in mated female worms was observed to be higher than that seen in female worms which had been separated *in vitro* (Fig. 1). The same trends were observed in male and female schistosomes, using a variety of specifically and uniformly labeled [^{14}C] glucoses (data not shown). Furthermore, under the conditions used in the present study, no significant variation in glucose uptake was observed in comparing 6 different groups of worms ranging from 50 to 97 days PI.

Unidirectional influx data were analyzed to

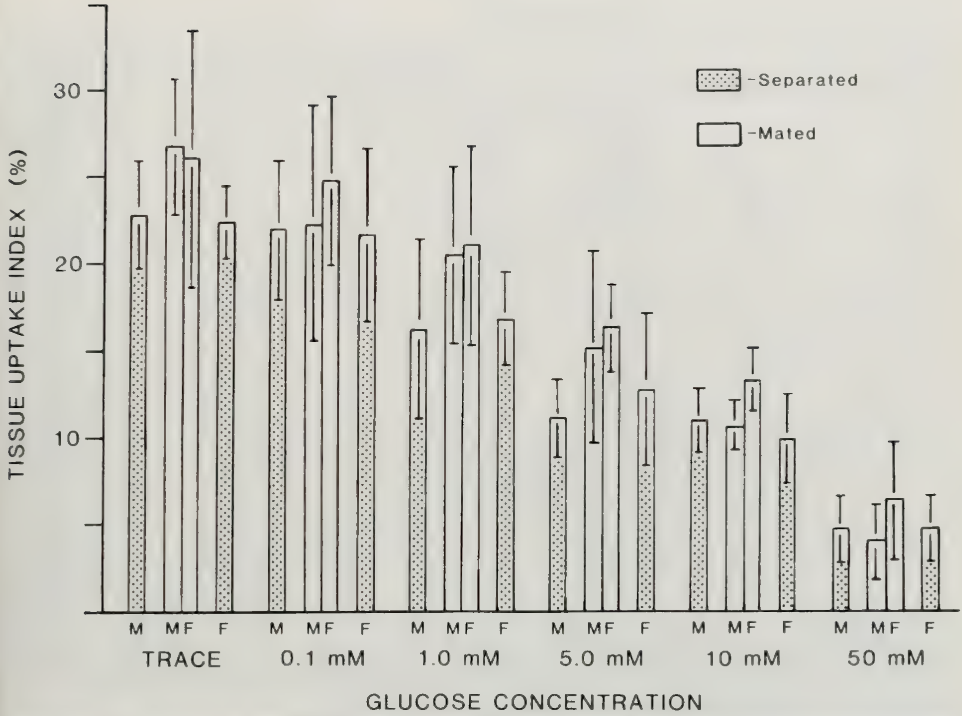

FIGURE 1. Tegumental uptake of [U-^{14}C] D-glucose in *Schistosoma mansoni* perfused from murine hosts 97 days postinfection. The tissue uptake index is proportional to [influx velocity/substrate concentration]. Over the range of glucose concentrations tested, alterations in the specific activity of the hexose were effected by use of a constant amount (ca. 2 μCi, 0.008 mM) of radioglucose, and final concentrations were adjusted through the addition of unlabeled solute. Note that in both males (M) and female (F), mated or copulative worms (open bars) generally take up greater quantities of glucose than do separated worms (stippled bars). Similar observations are apparent when different ages of schistosomes or different [^{14}C] glucoses are employed (see Table I). n = 8 for each $\bar{x} \pm$ SD.

estimate the diffusion component (K_d), as well as the half-saturation constant (K_m) and maximal velocity (V_{max}) of the facilitated diffusion system. In 2 instances, regression analysis suggested that the diffusion component in separated worms was so small as to be not significantly different from 0 (Table I). However, the typical pattern observed was that glucose uptake could be characterized by a diffusion component and a kinetically definable carrier-mediated transport mechanism. In any given group of worms examined, characteristic trends were apparent despite the intergroup variation for each parameter. For example, the diffusion component of mated female schistosomes was greater than that estimated for unmated females or males regardless of mating status (in each of the 6 age groups analyzed in Table I). The half-saturation constant in the copulative female appeared to be a higher-affinity (lower K_m) transport mechanism than observed for separated females or males (Table I), but as discussed below, these differences are not significant. In male schistosomes, the V_{max} was generally observed to be greater in copulative than in separated worms. In contrast, the estimated maximal velocity of glucose uptake was 2–5 times greater in separated than copulative female schistosomes (Table I). The permeability × surface area products were typically higher in mated than comparable separated worms, and this generalization applies to both males and females (Table I). Thus, tegumental permeability was directly proportional to observed tissue uptake indices. Furthermore, the schistosome tegumental permeability to glucose compares favorably to that of the host liver cell. In mated *S. mansoni*, PS = 1.4–3.8 μl/mg worm water/min (Table I), and in the mouse liver PS =

TABLE I. *Glucose uptake kinetics in* Schistosoma mansoni.

Glucose label	Worm age (d)	Sex*/mating	K_m† (mM)	V_{max} (nmol·min⁻¹/mg water⁻¹)	V_{max}‡	K_d (μl·mg water⁻¹/min⁻¹)	PS (μl mg water⁻¹/min⁻¹)
[2-¹⁴C]	55	F, P	0.95 ± 0.49	1.98 ± 0.96	7.4	0.02 ± 0.15	2.10 ± 0.26
		M, P	1.99 ± 0.97	2.84 ± 1.20	17.6	0§	1.43 ± 0.16
		F, U	1.07 ± 0.36	2.34 ± 0.71	10.3	0§	2.18 ± 0.15
		M, U	0.68 ± 0.19	0.93 ± 0.23	5.1	0§	1.37 ± 0.09
[6-¹⁴C]	57	F, P	1.43 ± 1.98	1.07 ± 1.45	4.0	0.79 ± 0.14	1.53 ± 0.18
		M, P	1.07 ± 0.42	1.36 ± 0.51	3.1	0.13 ± 0.07	1.39 ± 0.10
		F, U	5.48 ± 3.36	5.74 ± 4.25	25.3	0.12 ± 0.17	1.17 ± 0.05
		M, U	1.41 ± 0.53	0.90 ± 0.36	2.3	0.29 ± 0.04	0.93 ± 0.04
[6-¹⁴C]	50	F, P	3.19 ± 2.54	2.55 ± 2.29	4.8	0.71 ± 0.13	1.51 ± 0.07
		M, P	1.07 ± 0.30	1.47 ± 0.46	3.4	0.07 ± 0.09	1.45 ± 0.05
		F, U	4.86 ± 3.86	5.21 ± 4.93	22.9	0.13 ± 0.21	1.20 ± 0.07
		M, U	1.37 ± 0.78	0.87 ± 0.52	2.3	0.30 ± 0.06	0.93 ± 0.05
[1-¹⁴C]	69	F, P	0.35 ± 0.40	0.55 ± 0.61	2.1	0.44 ± 0.28	2.02 ± 0.46
		M, P	0.51 ± 0.33	0.57 ± 0.35	1.3	0.37 ± 0.10	1.49 ± 0.17
		F, U	3.56 ± 2.44	1.18 ± 0.83	20.3	0.01 ± 0.04	0.34 ± 0.03
		M, U	0.15 ± 0.06	0.30 ± 0.09	0.8	0.14 ± 0.07	2.12 ± 0.35
[1-¹⁴C]	58	F, P	2.31 ± 2.57	3.00 ± 3.98	1.1	0.57 ± 0.40	1.87 ± 0.16
		M, P	0.86 ± 0.66	1.80 ± 1.33	4.1	0.32 ± 0.30	2.41 ± 0.36
		F, U	1.31 ± 0.11	2.14 ± 0.16	9.4	0§	1.64 ± 0.03
		M, U	1.52 ± 0.10	1.67 ± 0.09	4.3	0§	1.11 ± 0.01
[U-¹⁴C]	97	F, P	0.28 ± 0.13	0.38 ± 0.17	1.4	1.06 ± 0.07	2.44 ± 0.10
		M, P	0.06 ± 0.05	0.11 ± 0.08	0.3	0.61 ± 0.12	2.39 ± 0.33
		F, U	0.89 ± 1.02	0.87 ± 0.94	3.8	0.36 ± 0.16	1.34 ± 0.25
		M, U	0.63 ± 0.42	0.88 ± 0.53	2.3	0.37 ± 0.10	1.76 ± 0.24

* F = female, M = male; P = paired (*in copula*), U = unpaired (separated).

† K_m = half-saturation constant; V_{max} = maximal influx velocity; K_d = diffusion component of uptake; PS = permeability × surface area product, where PS = $V_{max}/K_m + K_d$. Regression estimates ± SE were derived from data in Figure 1 for 97-day worms or from similar studies in which saturable uptake was determined from at least 5 glucose concentrations and multiple samples (n = 6–8) for each concentration tested.

‡ V_{max} converted to dry weight units = nmol/mg dry weight/min.

§ <0.0001, and not significantly different from 0.

2.8 μl/mg wet weight/min (Cornford et al., 1980) or approximately 2 μl/mg tissue water/min.

To test for these trends, across-experiment average estimates of the kinetic constants were obtained, and the *P*-values for gender-specific, mating-specific, and gender-mating effects determined from repeated measures analysis of variance (Table II); predicted influx velocities as a function of substrate concentrations are presented in Figure 2. Male–female differences in half-saturation constant ($P = 0.02$) and maximal velocity ($P = 0.004$) were highly significant (Table II). Mating status did not impart a significant effect upon K_m or V_{max} (Table II), perhaps in part because of the large variability of the individual parameter estimates (see Table I). However, there is a significant ($P = 0.01$) effect of mating on tegumental permeability (PS) and the V_{max}/K_m ratio.

The uptake of [¹⁴C] 3-O-methylglucose can be competitively inhibited by unlabeled glucose, in a concentration-dependent manner (Fig. 3). The mean of each of the kinetic constants (from Table II) is listed in Table III. The observation that the half-saturation constants for glucose inhibition of [¹⁴C] 3-O-methylglucose influx were not markedly different from the K_m's of glucose uptake suggests that these 2 hexoses shared a common transporter in copulative and separated males and females. Because 3-O-methylglucose is not metabolized by schistosomes (Isseroff et al., 1972), the similarity between K_i's and K_m's in Table III indicate that the kinetic parameters defined in our study were indeed characteristics of glucose influx, and not a measure of transport plus the metabolism of glucose.

The glucose analogs, 2-deoxyglucose (2-DG) and 3-O-methylglucose (3-O-MG), were transported across male and female schistosome surfaces by saturable, facilitated diffusion mechanisms (Table IV). The fact that uptake of both of these hexoses could be inhibited by D-glucose in cross-competition experiments (Fig. 3, Table IV) indicates that they share a common trans-

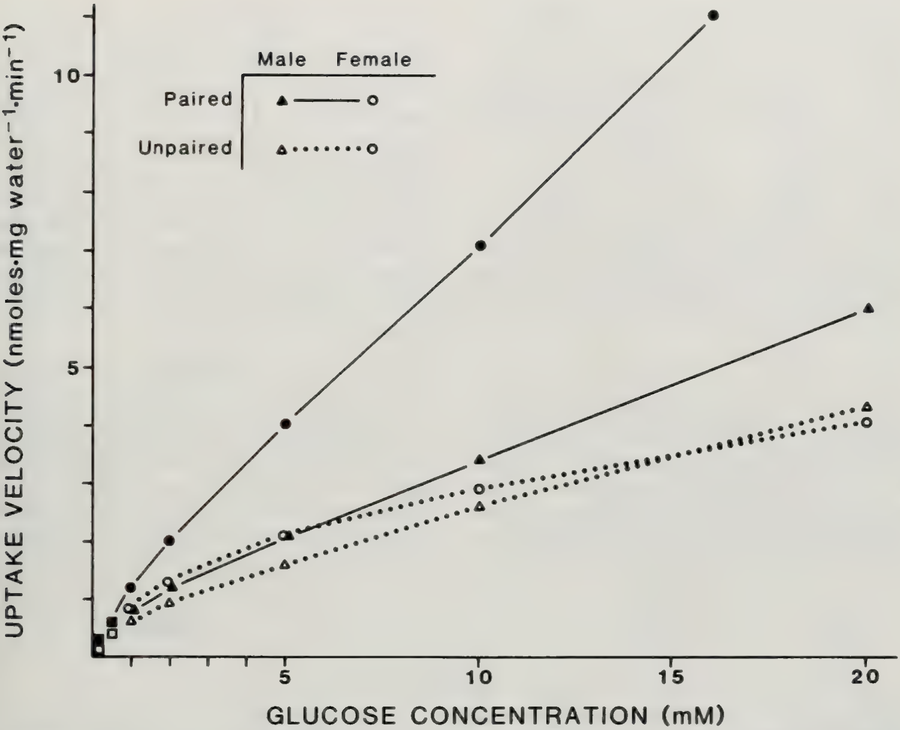

FIGURE 2. Predicted uptake velocities of glucose in male and female *S. mansoni* as a function of mating status. Uptake velocities were derived from mean transporter kinetic constants derived from studies of 6 different age-groups of schistosomes. Note that paired schistosomes (solid lines) have a greater requirement for glucose than seen in unpaired worms (broken lines). Variances in mean kinetic constants are tabulated elsewhere (Table II). Error bars have been omitted for clarity. For the data collected, the range of coefficients of variation in unpaired males (10.9–34.0%) and paired males (13.0–30.8%) was higher than in unpaired females (7.2–26.7%) and paired females (11.4–20.4%).

TABLE II. *Mean glucose transporter kinetics in* S. mansoni.

Sex	Mating status	K_m* (mM)	V_{max} (nmol/min/mg water)	K_d (μl/min/mg water)	V_{max}/K_m (μl/min/mg water)	PS (μl/min/mg water)
F	Paired	0.99 ± 0.40	1.22 ± 0.42	0.60 ± 0.14	1.31 ± 0.21	1.91 ± 0.15
M	Paired	0.63 ± 0.31	0.93 ± 0.44	0.25 ± 0.09	1.51 ± 0.14	1.76 ± 0.20
F	Unpaired	2.21 ± 0.73	2.32 ± 0.72	0.10 ± 0.06	1.21 ± 0.26	1.31 ± 0.25
M	Unpaired	0.76 ± 0.27	0.83 ± 0.19	0.18 ± 0.07	1.19 ± 0.21	1.37 ± 0.20
P-values from repeated measures analysis of variance†						
Sex effect		0.02	0.0004	0.10	0.66	0.81
Mating effect		0.17	0.4	0.03	0.01	0.01
Interaction		0.4	0.2	0.02	0.7	0.7

* K_m, V_{max} = geometric mean (antilog of mean of logs). K_d, V_{max}/K_m, PS = arithmetic mean; F = female, M = male.

† ANOVA *P*-values based on logarithms for K_m and V_{max}, and square root for K_d. Geometric means ± asymptotic SE and arithmetic means ± SE are derived from data in Table I. V_{max}/K_m ratios are an indicator of transporter efficiency (or permeability, independent of the diffusion component); higher V_{max}/K_m ratios characterize transporters with increased influx rates at substrate concentrations near that of the K_m. PS products characterize tegumental permeability at reduced substrate concentrations. Note that the gender differences for K_m and V_{max} are significant ($P < 0.05$), and that V_{max}/K_m and PS products are significantly greater in mated than in separated schistosomes.

TABLE III. *Half-saturation constants of glucose inhibition (K_i) on influx of [^{14}C] 3-O-methylglucose in* Schistosoma mansoni.*

Sex	Mating status	K_i (mM)	Glucose K_m (mM)
F	Paired	1.03 ± 0.84	0.99 ± 0.40
M	Paired	3.74 ± 2.65	0.63 ± 0.31
F	Unpaired	1.58 ± 0.92	2.21 ± 0.73
M	Unpaired	1.63 ± 0.45	0.76 ± 0.27

* The regression estimates ± SE of the half-saturation constants of inhibition (K_i) were derived from data in Figure 3. Glucose K_m values, for comparison, are from Table II. Similarities between the K_i of glucose-inhibitable [^{14}C] 3-O-methylglucose influx and the half-saturation constant for glucose suggest these 2 hexoses share a common transporter.

porter in male and female schistosomes. Kinetic analyses (comparing K_m's listed in Tables II and VI) indicated that 2-DG appeared to have a higher affinity (lower half-saturation constant) for the hexose carrier than did glucose. The increases of 2-DG influx seen in mated schistosomes are apparent in Figure 4. Conversely, 3-O-MG uptake was generally characterized by a lower-affinity (higher K_m, Table V) mechanism than seen for glucose (Table II) or 2-DG (Table VI). In addition, the uptake of 3-O-MG (graphically presented in Fig. 5) was best characterized as a single component system. That is, the uptake of this sugar was primarily via the saturable transport system (Table V), without a significant K_d (except for paired male schistosomes). The permeability (as indicated by the PS products) of male and female schistosomal surfaces for 3-O-MG was also significantly increased in mated schistosomes (Table V).

DISCUSSION

The male partner, rather than the environmental plasma or medium, supplies glucose to the copulative female schistosome (Cornford and Huot, 1981). It has also been suggested that a significant fraction, if not all, of the glucose obtained by the female is supplied by the male partner (Rogers and Bueding, 1975; Cornford and

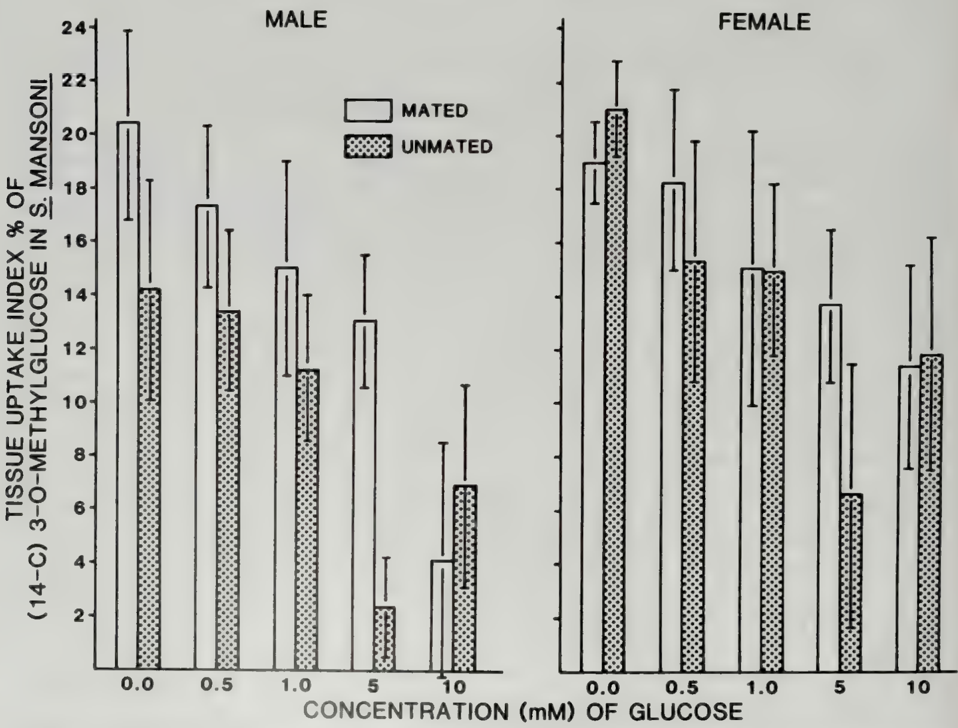

FIGURE 3. Glucose inhibition of tegumental uptake of [U-^{14}C] 3-O-methylglucose in *Schistosoma mansoni* perfused from mice 49 days PI. The concentration-dependent inhibition of tracer concentrations (about 2 μCi, 0.030 mM) of 3-O-methylglucose uptake by unlabeled glucose confirms that in both male and female schistosomes, these 2 sugars enter via the same transporter. n = 8 for each $\bar{x}$ ± SD.

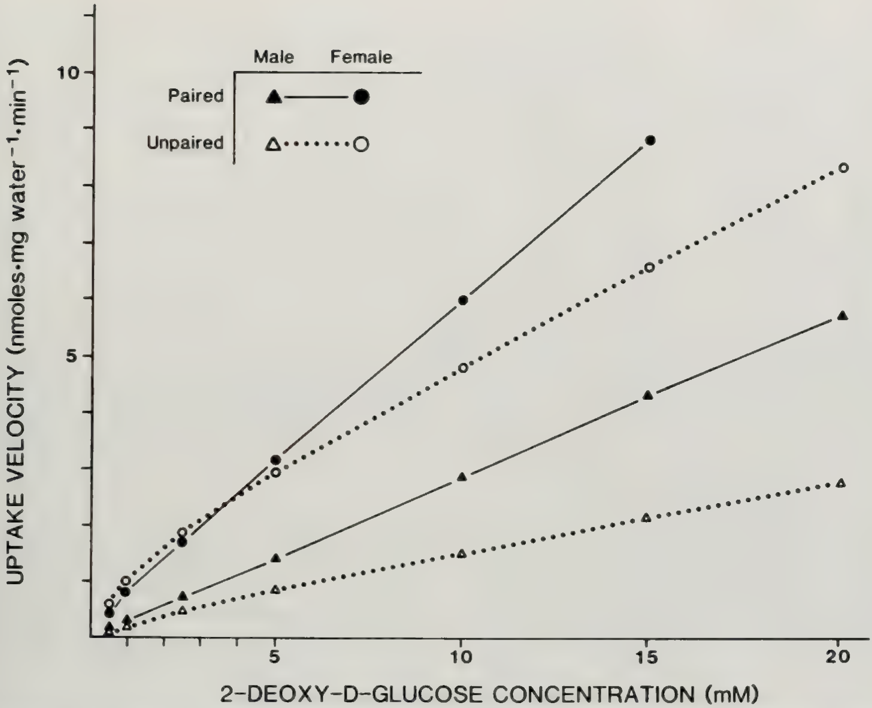

FIGURE 4. Gender- and mating-specific comparison of 2-deoxy-D-glucose influx in *S. mansoni*. Predicted uptake velocities were derived from mean transporter kinetic constants (Table VI). Standard errors are listed elsewhere (Table VI). An additional measure of variance at different solute concentrations is given by the standard deviations (of data prior to transformation) listed in Table IV.

Fitzpatrick, 1985). Whereas the uptake mechanisms defined for copulative female schistosomes established that transport mechanisms are present, the apparent half-saturation constants and maximal velocities defined (Tables I, II) assume the source of hexoses to be the medium, rather than the male partner.

Attempts were made to calculate uptake velocities in mated female schistosomes using, as the substrate concentration, the (internal) free glucose concentration in the male partner (data not shown). Based on this assumption, the diffusion component and permeability (PS products) are unchanged, and the K_m and V_{max} shifted in the same direction (i.e., a 5-fold reduction). The apparent 20% reduction in half-saturation constants and maximal uptake velocities were directly proportional to the gradient in free glucose which exists between the environment and the subtegumental tissues of the mated male schistosome (Cornford and Fitzpatrick, 1985). However, because the estimates of free glucose concentration are based on equilibrium (steady-state) conditions, and the transporter kinetics are derived from initial rate measurements, the data in Table II are assumed to best represent transporter kinetic constants of the mated female schistosome.

Glucose transport across biological membranes is typically described by operational constants that include the maximal transport velocity (variously referred to as T_{max}, J_{max}, or V_{max}), and the half-saturation constant (K_m or K_t). The nonsaturable uptake component (K_d) is typically small relative to the mediated fraction, and thus workers do not always numerically define this constant. The maximal velocity varies as a function of (a) the number of transporter sites per unit of membrane, and (b) the intrinsic activity, or rate at which glucose penetrates through the membrane. The half-saturation constant is a function of the probability of finding glucose molecules bound to the transporter, rather than free in the medium or plasma (Le Fevre, 1975).

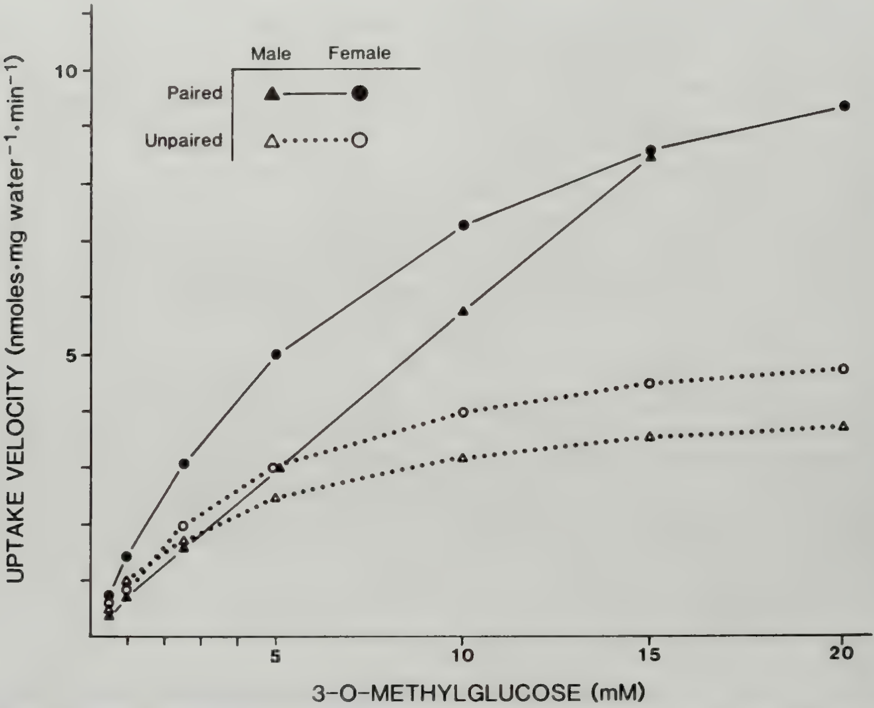

FIGURE 5. Gender- and mating-specific comparison of predicted 3-O-methylglucose influx in 45-day PI *S. mansoni* (see Table IV). Note that as seen for glucose (Fig. 2), paired schistosomes transport increased quantities of 3-O-methylglucose. Standard errors are listed in Table V, and the variance of data prior to transformation is given by the standard deviations listed in Table IV.

Because the membrane surface areas exposed by male and female schistosomes differ greatly with mating state, the comparison of transport constants in mated and separated male and female schistosomes provides some information regarding the anatomic site(s) of glucose transport systems.

The present studies indicate that glucose permeability in male and female *S. mansoni* is quite responsive to the copulative state. The observation that significantly higher V_{max}/K_m ratios and PS products were seen in mated than in unmated schistosomes (Table II) suggests that the presence of a female in the gynecophoral canal of the male modifies transporter function, and perhaps the apparent diffusion component. Presumably, the increase in the V_{max}/K_m ratio is achieved either by increasing the activity, or number, of transport sites on the external male tegument. In adipocytes, for example, it has been established that insulin-dependent increases in the V_{max} of glucose transport are attributable to an increase in the number of transporter sites, and that transporters could be translocated from intracellular sites (Simpson and Cushman, 1985). The mechanism responsible for higher PS products (i.e., V_{max}/K_m ratios) in mated than in unmated male schistosomes (Table II) is not defined, but it occurs when the gynecophoral surface is physically blocked by the female. Because the surface of the gynecophoral canal is exposed to medium in unmated males, but is in intimate contact with the tegument of the female partner *in copula*, the copulative state dramatically reduces the functional surface area of the schistosome. Thus, the increase in PS products for glucose occurs concomitantly with a physical reduction in surface area. Our data therefore indirectly support the suggestion of Fripp (1967) that glucose uptake in male schistosomes occurs primarily at the dorsal tegument and not along the surface of the gynecophoral canal.

It is now established that development of vitelline glands in female schistosomes requires

Table IV. *Tegumental uptake of glucose analogs in* Schistosoma mansoni.

Age (days)	[^{14}C] hexose	Solute	Sex, mating	Trace (<0.05 mM)	TUI (%) at various solute concentrations* 0.1 mM	0.5 mM	1.0 mM	5 mM	10 mM
55	2-DG	2-DG	M, P	20.7 ± 3.1	—	12.3 ± 4.2	—	7.2 ± 5.1	5.3 ± 2.1†
			M, U	18.4 ± 2.0	—	14.4 ± 3.7	—	7.8 ± 1.1	4.2 ± 3.1†
			F, P	18.2 ± 3.7	—	11.6 ± 3.3	—	10.3 ± 3.2	8.6 ± 3.3†
			F, U	23.0 ± 1.9	—	19.4 ± 1.4	—	12.6 ± 1.6	4.7 ± 4.0†
45	3-O-MG	3-O-MG	M, P	20.0 ± 3.2	16.5 ± 0.8	—	10.8 ± 5.1	10.2 ± 2.9	6.5 ± 3.5
			M, U	18.2 ± 4.5	14.0 ± 2.2	—	13.7 ± 1.8	8.2 ± 1.7	3.5 ± 2.0
			F, P	22.3 ± 2.0	20.6 ± 4.3	—	19.7 ± 2.5	15.0 ± 4.0	11.0 ± 3.2
			F, U	25.1 ± 5.9	19.5 ± 0.9	—	18.7 ± 1.8	12.2 ± 1.4	6.6 ± 4.9
93	Glucose	2-DG	M, P	21.0 ± 3.9	21.7 ± 4.5‡	—	13.3 ± 2.9§	—	4.1 ± 1.9†
			M, U	13.8 ± 2.9	12.5 ± 2.4‡	—	6.3 ± 1.6§	—	1.1 ± 1.0†
			F, P	18.4 ± 3.8	13.5 ± 2.9‡	—	8.2 ± 3.4§	—	7.0 ± 1.8†
			F, U	19.4 ± 3.9	18.3 ± 2.8‡	—	10.3 ± 2.9§	—	4.4 ± 2.6†

* Over the range of concentrations tested, alterations in the specific activity of the hexose were effected by using a constant amount (the trace concentration, approximately 2 μCi) of the [^{14}C] hexose and adjusting final concentrations by the addition of unlabeled solute.
† 20 mM solute concentration.
‡ 0.2 mM solute concentration.
§ 2.0 mM solute concentration.

physical copulative contact of the female with a male partner. A size reduction, with concomitant regression in the reproductive system is seen when mature females are isolated from their male partners (Popiel and Basch, 1984; Popiel et al., 1984). Thus, it is possible that the increased requirement for glucose observed for mated worms in the present study is attributable to reproductive functions, perhaps involving both energy production and biosynthesis. Because serotonin-dependent increases in glucose uptake in *S. mansoni* are characterized by subtle shifts in the distribution of isotope from [^{14}C] glucose, which suggest a priority of energy generation over biosynthesis (Rahman et al., 1985), one might speculate that similar shifts occur in response to mating.

Most of the external surfaces of mated females are surrounded by the gynecophoral canal of the male partners; because the free glucose content of males is approximately one-fifth that of the surrounding medium, and assuming the media does not equilibrate in the remaining male/female interface in the canal, these females have a reduced access to glucose. The kinetic constants thus defined for copulative schistosomes may be relatively more imprecise and are complicated by male-to-female transfer of the radiolabeled substrate.

The half-saturation constants derived in our study are in agreement with previous reports. Values previously reported were: pairs = 0.8 mM (Uglem and Read, 1975), separated males = 0.56 mM and separated females = 0.57 mM (Podesta and Dean, 1982a), in contrast to the 9.0 mM reported by Isseroff et al. (1972). (The data presented by Isseroff et al. [1972] suggest a half-saturation constant of about 1.5 mM.) The rate of glucose utilization by paired schistosomes is about 6.3 nmoles/min/mg dry weight (Shapiro and Talalay, 1982). The validity of our estimates of uptake velocities (per unit dry weight) in paired worms (Table I) is suggested by the consistency with this utilization rate.

The suggestion that glucose uptake could be higher in separated males and females than for

Table V. *Kinetics of 3-O-methylglucose in* Schistosoma mansoni.*

Glucose	Worm age (days)	Sex mating	K_m	V_{max}	K_d	PS
3-O-MG	45	F, P	8.40 ± 2.56	13.41 ± 3.82	0†	1.60 ± 0.06
		M, P	0.29 ± 0.24	0.27 ± 0.21	0.55 ± 0.09	1.50 ± 0.15
		F, U	5.01 ± 1.11	6.00 ± 1.18	0†	1.20 ± 0.06
		M, U	4.02 ± 0.95	4.51 ± 0.97	0†	1.12 ± 0.06

* Units as described in Table I. K_m = mM, V_{max} = nmol/mg worm water/min, K_d and PS = μl/mg worm water/min. Values listed are regression estimates ± SE.
† <0.0001, and not significantly different from 0.

TABLE VI. *Comparison of 2-deoxy-D-glucose uptake kinetics and 2-deoxy-D-glucose inhibition of glucose uptake.**

			Mean 2-deoxy-D-glucose kinetic constants			
Sex	Mating status	K_i (mM)	K_m	V_{max}	K_d	PS
F	Paired	0.21 ± 0.01	0.45 ± 0.18	0.36 ± 0.17	0.57 ± 0.11	1.35 ± 0.04
M	Paired	2.85 ± 1.23	0.094 ± 0.12	0.027 ± 0.015	0.29 ± 0.27	1.18 ± 1.11
F	Unpaired	1.53 ± 0.23	1.28 ± 0.56	1.52 ± 1.45	0.35 ± 0.23	1.70 ± 0.41
M	Unpaired	1.58 ± 0.30	1.36 ± 1.31	0.28 ± 0.33	0.13 ± 0.05	0.33 ± 0.02

* Similarities in the 2-deoxyglucose-inhibitable influx of [^{14}C] D-glucose (i.e., the K_i) and the half-saturation constant (K_m) of 2-deoxy-D-glucose influx suggest that these hexoses share a common transporter. Mean ± SE 2-deoxyglucose kinetics were derived from studies of schistosomes perfused 55, 60, and 63 days PI.

equivalent worm pairs (Mercer and Chappell, 1986) is not consistent with the present results, or previous studies (Cornford and Huot, 1981; Cornford, 1982, 1986). Mercer and Chappell (1986) incubated their schistosomes for 2 min in [^{3}H] glucose, then washed their worms with 3 rinses of cold normal saline. Glucose "uptake" was subsequently determined by the extraction of free isotope with 70% ethanol as employed by Uglem and Read (1975). The saline rinse undoubtedly removes a large fraction of the intra-worm free glucose. The half-time of washout of tritiated water from schistosomes in saline solutions, for example, is less than 10 sec. Further evidence for errors in Mercer and Chappell's (1986) glucose uptake estimates are suggested by the fact that their tabulated estimates of uptake (1–50 pmoles/min/mg dry weight) are orders of magnitude less than established rates of glucose utilization by mansonian schistosomes (6.3 nmoles/min/mg dry weight; Shapiro and Talalay, 1982).

In terms of the methods available for measuring glucose uptake, steady-state measurements are compromised because of metabolism of the substrate, whereas initial rate measurements require precise estimation of the effects of unstirred layers (Podesta and Dean, 1982a). In the present study, the short time periods employed and the consistency of the data obtained with the variety of specifically labeled [^{14}C] glucoses collectively suggest that our measurements reflect transport, as opposed to transport plus metabolism. Furthermore, the internal consistency between glucose kinetics and cross-inhibition studies (Table III) with [^{14}C] 3-O-methylglucose (which is transported, but not metabolized by schistosomes) further support this concept.

In the case of 2-DG inhibition of glucose uptake, the K_i's estimated for 2-DG (0.2–2.5 mM) inhibition exceeded the K_m's of the 2-DG transporter (0.3–0.9 mM). As indicated in Table IV, the "tracer" concentration of [1-^{14}C] glucose employed was 0.03 mM. Given that the K_m's for 2-DG uptake range from 0.1 to 1.4 mM (Table VI), the amount of ^{14}C substrate employed greatly exceeds a tracer (<1%) concentration. Although it was established that 2-DG and glucose share a similar transporter in male and female schistosomes, the affinity of 2-DG for this locus was best estimated from 2-DG self-inhibition studies (Table VI).

The K_m for glucose transport in mated female schistosomes was estimated to be 1.0 mM (Table II). In *S. mansoni* homogenates, hexokinase is the rate-limiting enzyme, and it has been suggested that in intact worms, glucose transport may be the rate-limiting step in glucose utilization (Shapiro and Talalay, 1982). Since the free glucose levels within male schistosomes are one-fifth that of the external medium (Cornford and Fitzpatrick, 1985), whenever plasma glucose levels fall below 5 mM, the free glucose level of the male will fall below 1.0 mM. For the copulative female partner, the external glucose concentration (i.e., the free glucose level in the male) will fall below the female's tegumental transporter K_m (ca. 1.0 mM). Under these conditions, internal glucose levels in the female will fall sharply and membrane transport (male-to-female transfer) may well become the rate-limiting step in glucose utilization. Further studies are needed to test this hypothesis.

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RESA-IFA ASSAY IN *PLASMODIUM FALCIPARUM* MALARIA, OBSERVATIONS ON RELATIONSHIP BETWEEN SERUM ANTIBODY TITERS, IMMUNITY, AND ANTIGENIC DIVERSITY

C. E. Contreras, J. I. Santiago, J. B. Jensen, I. J. Udeinya*, R. Bayoumi†, D. D. Kennedy, and P. Druilhe‡

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

ABSTRACT: RESA-IFA assays were conducted using 63 adult sera from 7 different malarious areas against 7 different strains of *Plasmodium falciparum*, and 28 children's sera against 3 different parasite strains. Generally, where immunity to malaria was high, there was little or no antigenic diversity among the different strains examined. However, where sera were collected from semi-immunes, or from children, some variation in the RESA-IFA endpoint titers was discernible. Correlation between antibody titers determined by RESA-IFA and *in vitro* parasite invasion inhibition was not complete. Sera having high RESA-IFA titers were predictably inhibitory; however, many sera having low RESA-IFA titers were as inhibitory as sera having high titers, indicating that antibodies with specificities different from the RESA may be as important, or more important, to clinical immunity to blood-stage infections.

The twin tragedies of broad-spectrum insecticide resistance in malaria-transmitting mosquitoes and the continuing spread of parasite drug resistance have significantly undermined global efforts for the control of malaria and justified the hundreds of recent reports on malaria immunology. Because most of these reports have sufficiently reviewed the problem, further elaboration here would be redundant. Many potential malaria vaccine candidates against the disease-inducing blood-stage infection have been discovered (Heidrich, 1986). One of these showing promise is a parasite-derived molecule inserted into the plasmalemma of *Plasmodium falciparum*-infected erythrocytes known as the Pf 155, or the "ring-infected erythrocyte surface antigen," or RESA (Brown et al., 1985). Interestingly, this antigen can be visualized by indirect immunofluorescence on glutaraldehyde-fixed, air-dried, ring-infected erythrocyte monolayers, constituting the RESA-IFA test (Perlmann et al., 1984). Furthermore, evidence has been reported correlating serum RESA titers, determined by RESA-IFA, to *in vitro* parasite invasion inhibition (Wahlin et al., 1984), and to clinical immunity to malaria in Liberia (Wahlgren et al., 1986). Several studies have cited limited evidence that the RESA antigen is highly conserved, with no evidence of antigenic variation or diversity (Coppel et al., 1984; Cowman et al., 1984; Perlmann et al., 1984; Wahlin et al., 1984; Wahlgren et al., 1986). Thus, if serum RESA concentrations prove to be a good correlation of anti-merozoite immunity, and if no antigenic diversity by different geographic strains of *P. falciparum* exist, the RESA-IFA test could become a valuable tool for epidemiologic studies on malaria immunity, and as a monitoring device for successful induction of clinical immunity by immunization during field trials on malaria blood-stage vaccines. To further investigate these possibilities, we examined 63 adult sera from 7 geographic regions against 7 different strains of *P. falciparum*. We also examined 28 sera from children, ages 2–13 yr, against 3 different parasite strains.

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* Department of Pharmacology and Therapeutics, School of Medicine, University of Nigeria, Enugu, Nigeria.

† Department of Biochemistry, Faculty of Medicine, University of Khartoum, Khartoum, Sudan.

‡ Parasitologie et Mycologie, Maladies Tropicales et Parasitaires, Assistance Publique Hôpitaux de Paris, 47, Boulevard de l'Hôpital-75561, Paris, Cédex 13, France.

MATERIALS AND METHODS

Parasites

We examined RESA-IFA fluorescence in 7 strains of *P. falciparum* collected from different geographic regions. These were: FCC_1, The People's Republic of China; FCR_3, The Gambia; FCR_8, Nigeria; $FCMSU_1$, Sudan; G-73, Irian Jaya, Indonesia; F-2382, Flores, Indonesia; and FCB, Colombia, South America. All of the parasites were well adjusted to culture, and some have been established for many years. Sera to be examined for *in vitro* anti-merozoite invasion inhibition were tested against strains FCB, $FCMSU_1$, and G-73, representing South American, African, and Southeast Asian strains, respectively.

Human malaria-immune sera

We examined sera from 63 adults living in malaria-endemic regions for RESA-IFA titers using the 7 strains of *P. falciparum* listed above. These sera were collected by J.B.J., or were gifts from individuals acknowledged, including sera from Nigeria: 17 samples, of which 4 were collected from urban Enugu and 13 from rural Anambra State villages; Sudan: 14 samples, of which 6 were collected from Sobat River area in Jonglei Province and 8 from villages south of Damazin, Blue Nile Province; Irian Jaya, Indonesia (Western New Guinea), 8 samples from Mapurajaya; Flores, Indonesia: 8 samples; Burkina Faso (Upper Volta): 8 samples; and Ecuador: 8 samples. Once it became apparent that sera from adults having low RESA-IFA titers showed some evidence of strain-specific antigenic diversity, we expanded our study to include sera collected from young children (presumably nonimmune, or semi-immune) living in hyperendemic villages south of Damazin, Sudan. We examined 28 sera from children between ages 2–13 yr against parasite strains FCR_3, $FCMSU_1$, and FCB.

RESA-IFA test

The ring-infected erythrocyte surface antigen, indirect immunofluorescent assay (RESA-IFA) was conducted using the method of Perlmann et al. (1984) with modifications. Briefly, highly synchronous ring-infected erythrocytes from the 7 different parasite strains were grown to 8–10% parasitemia, and used to prepare the test slides using blank 8-well multitest *Toxoplasma* slides from Bellco Glass Co. (Vineland, New Jersey). Fifteen μl of a 2% suspension of infected RBC's were added to each well after charging the slide with the bicarbonate coating buffer. After 30 min settling time, the slides were washed by dipping in Tris-buffered Hanks' solution, fixed 10 sec in 1% glutaraldehyde, rinsed in PBS, refixed 10 sec in fresh 1% glutaraldehyde, thoroughly washed in distilled water, and immediately air-dried. Slides were packaged with desiccant and frozen at −40 C until used. Sera were titered by serial dilutions, applied to the RESA slides, and processed for immunofluorescence using Biotin–Avidin-conjugated anti-human IgG (Vector Laboratories, Burlingame, California, affinity-purified biotinylated anti-human IgG, 30μg/ml and fluorescein-conjugated Avidin, 50 μg/ml, working concentrations, respectively).

We were concerned that the parasite-derived antigen in the erythrocyte membrane might be dissipated over time, affecting the titration endpoints in some parasite strains. Thus, we tested the stability of the antigen-containing membrane by synchronizing cultures to a 4-hr age differential using a combination of the sorbitol (Lambros and Vanderberg, 1979) and gelatin flotation techniques (Jensen, 1978) and examined titered sera in plates prepared from 0–4, 4–8, 8–12, 12–16, 16–20, and 20–24 postinvasion-infected cells.

Standard schizont-IFA test

Parasite cultures containing a high percentage of mature schizont-infected erythrocytes were spread as thin films, air-dried, and hemoglobin removed by dipping in 0.3 N HCl. Eight wells were created on the slide using commercial, felt-tipped fingernail polish applicators. Serially diluted sera were applied to the wells, incubated 30 min, washed 3× in PBS, and after overlaying the films with fluorescein-conjugated goat anti-human IgG (1:10 dilution of commercial stock, Cappel Laboratories, Cochranville, Pennsylvania) were reincubated for 30 min and rewashed 3× in PBS. The protocols for the RESA-IFA and standard IFA use different conjugates in keeping with procedures developed for these assays over the years. The standard IFA is generally not sensitive enough for the RESA assay, and using Biotin–Avidin conjugates would give higher standard IFA titers, thus making comparisons of our data with previous reports more difficult.

In vitro merozoite invasion inhibition

To determine whether RESA-IFA titers correlated with *in vitro* inhibition of merozoite invasion, we examined selected sera using strains $FCMSU_1$, FCB, and G-73 by a modification of Vande Waa et al. (1984). Briefly, synchronous, multinucleated schizonts were concentrated by gelatin flotation (Jensen, 1978) and diluted to 2% parasitemia with freshly washed O^+ erythrocytes and returned to culture at 2% hematocrit in 96-well microculture plates in RPMI-1640 medium containing 25% v/v dialyzed immune sera. After allowing 5 hr for merozoite invasion to proceed in the presence of the immune sera, the medium was aspirated from the plates that were washed with 5% sorbitol to eliminate all stages except the newly invaded rings, washed 2× with medium, and returned to culture with RPMI-1640 medium containing [^{3}H] hypoxanthine (1 μCi/μl) for 24 hr, then harvested onto glass-fiber filter strips. Parasitemias were determined using liquid scintillation spectrometry.

RESULTS

The analysis of 63 adult and 28 pediatric sera against the different parasite strains resulted in 525 RESA-IFA and 63 standard IFA assays—data too voluminous to detail in this report. Hence, we have summarized these data in Table I, with some analytical comments below. Among the Nigerian serum samples only 1 of 17 was RESA-IFA-negative for all parasite strains. Of the remaining, only 1 had a 2-dilution differential in RESA-IFA endpoint titers; all the rest being consistently within the acceptable 1-dilution range. The sera from Sudan were collected from 2 different regions. Sera from the remote Jonglei Province had 1 sample with no RESA-IFA antibody, but only 1 sample showed no antigenic diversity. Four of 6 sera showed significant variation ranging from 0 to 1:80, and 0 to 1:640, with the remaining samples having a greater than 2-dilution differential (1:40 through 1:640; 1:320 through 1:2,560). Sudanese sera from southern Blue Nile Province were remarkably consistent with regard to RESA-IFA titers between the 7 different parasite strains. Two showed a 2-dilu-

TABLE I. *A summary of 525 RESA-IFA and 63 schizont-IFA assays conducted in this study.**

Sera	n	RESA-IFA†			Schizont-IFA†	
		Range	Median	Variation	Range	Median
Nigeria	17	0–10,240	640	1/17‡	640–20,480	5,120
Sudan-J	6	0–2,560	80	4/6	160–10,240	2,560
Sudan-BN	8	0–10,240	1,280	2/8‡	160–20,480	2,560
Irian Jaya	8	2,560–10,240	5,120	0/8	5,120–20,480	10,240
Flores	8	1,280–5,120	2,560	0/8‡	1,280–20,480	5,120
Burkina Faso	8	0–640	160	1/8	1,280–2,560	1,280
Ecuador	8	0–2,560	80	7/8	20–2,560	320
Children§	28	0–1,280	128‖	13/19#	ND¶	

* Of all sera examined, only 3 different sets showed strong evidence of strain variation in RESA-IFA titers. Adult sera were examined against 7 different strains of *Plasmodium falciparum*, the children's sera against 3 strains.

† Values represent the reciprocals of endpoint titers.

‡ Variation in RESA titers did not exceed 2 dilutions.

§ Sera examined against 3 different parasite strains.

‖ Represents sera positive for 1 or more strains.

Since 9/28 had no RESA antibody, 13/19 showed some variation.

¶ Schizont-IFA titers not done

tion (but not more) difference in endpoint titers against 2 of 7 strains.

Sera were collected from 2 distinct localities in Indonesia: Irian Jaya (Western New Guinea) and the island of Flores. The samples from Irian Jaya were remarkable in that their RESA-IFA titers were extremely high, always consistent, and nearly always within the 2 dilutions of their schizont IFA titers. The samples from Flores were generally similar, but had slightly lower RESA-IFA and schizont IFA titers. Except for 1 serum from Flores, all sera from Indonesia were within the acceptable range, indicating no RESA-IFA variation when tested against the 7 different parasite strains.

Only 1 serum sample from Burkina Faso (formerly Upper Volta) was without some RESA-IFA antibody, and only 1 showed any antigenic variation. The RESA-IFA titers were significantly lower than those seen in sera from Nigeria and Indonesia, but notwithstanding their lower endpoint titers, they were generally uniform, ranging between 1:160 and 1:640, suggesting low but consistent malaria exposure. Serum samples from Ecuador had the greatest degree of diversity in endpoint titers against the different parasite strains, suggesting the greatest degree of antibody/antigen variation. One sample was negative for all strains examined (notwithstanding its schizont IFA titer of 1:320). Of the remaining 7 samples, 6 had negative RESA-IFA titers for some parasite strains, but titers up to 1:160; 1:320; and 1:640 against other strains. One sample varied between an endpoint titer of 1:320 with 1 strain to 1:2,560 when a different parasite strain was used for the assay.

Of the 28 serum samples collected from Sudanese children, 9 (32%) had no RESA-IFA antibody detectable against any of the parasite strains examined. Of the remaining samples, no relationship could be discerned between age and RESA-IFA titers—being randomly distributed between 1:40 and 1:1,280. Six samples (30%) showed no significant variation in RESA-IFA titers among the parasite strains examined. Seven samples were negative for 1 or more samples, while showing low titers between 1:4 and 1:16 for other strains. The remaining 6 samples showed significant variation ranging between 1:4 through 1:16 for some samples through 1:160 through 1:1,280 for other samples.

We selected several sera from different regions having a wide range of RESA-IFA titers for testing of merozoite invasion inhibition *in vitro* using parasite strains FCB, $FCMSU_1$, and G-73. The results of these assays are found in Figure 1. Generally, there was a 1-dimensional correlation between RESA-IFA endpoint titers and *in vitro* merozoite invasion inhibition. Thus, when the RESA-IFA titers were equal to or greater than 1:1,280, inhibition of merozoite invasion into erythrocytes *in vitro* was greater than 50%. However, several sera having low RESA-IFA titers were nonetheless highly inhibitory to merozoite invasion. For example, several sera with RESA-IFA titers less than 1:160 inhibited merozoite invasion by greater than 50%; 1 serum having a RESA-IFA titer of 1:40 blocked merozoite invasion by greater than 80%.

Using highly synchronous parasite cultures having a total age differential of less than 4 hr, RESA slides were made from parasites ranging

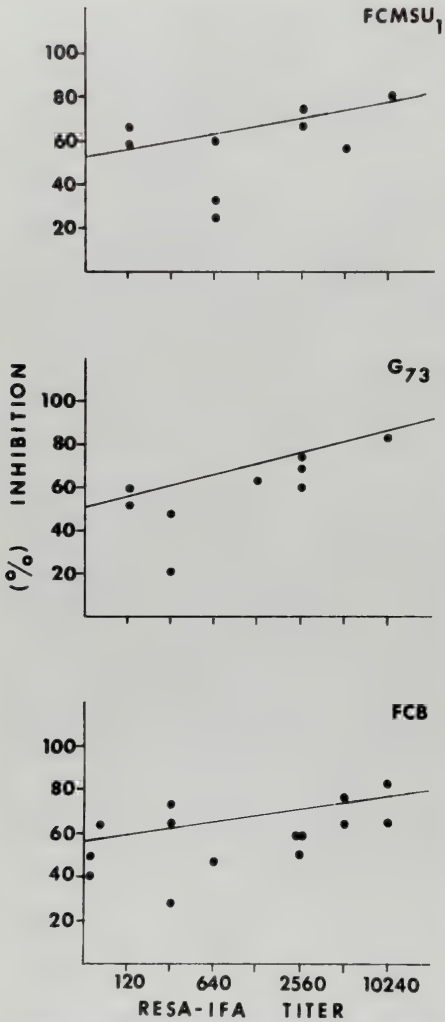

FIGURE 1. *In vitro* merozoite invasion inhibition by adult sera against 3 different strains of *Plasmodium falciparum*. Generally, as the RESA-IFA titers increased, the inhibition of merozoite invasion into erythrocytes also increased. However, several sera having low titers of RESA antibodies were as inhibitory, or more so, than sera having high RESA-IFA titers. The correlation coefficients for strains $FCMSU_1$, G-73, and FCB were 0.540 ($P < 0.10$), 0.682 ($P < 0.05$), and 0.465 ($P < 0.10$), respectively; representing values that were significant only for 1 set of experiments, and overall were not particularly impressive.

between 0 and 4 hr postmerozoite invasion and 24 and 28 hr postinvasion. Four sera having RESA-IFA titers between 1:1,280 and 1:20,480 were examined in 7 different parasite age groups. The RESA-IFA endpoint titers did not vary in any sample irrespective of the age of the parasites through 28 hr postmerozoite invasion. However, after 32 hr, some sera began to vary in their titers, but generally erythrocyte surface fluorescence continued well into the schizont development, becoming inconsistent once karyokinesis had begun. From a practical standpoint, the sorbitol synchronization technique of Lambros and Vanderberg (1979), which produces an 18-hr synchrony, is sufficient for the preparation of RESA-IFA slides.

DISCUSSION

Our results suggest that the parasite antigen found inserted into the plasmalemma of newly *P. falciparum*-invaded erythrocytes has some antigenic diversity, but the significance of this situation is yet unclear. Our sera can be divided into 4 classifications: (1) adult sera from areas where malaria transmission is hyperendemic to holoendemic and reasonably stable (Nigeria, Indonesia, Sudan, Blue Nile Province, and Burkina Faso); (2) hyperendemic unstable but geographically remote, requiring 4 hr flying time in small aircraft over trackless wilderness (Sudan, Jonglei Province); (3) mesoendemic (Ecuador), and (4) pediatric sera collected in hyperendemic Sudan, Blue Nile Province. Generally, when sera from region (1) were tested against the 7 different parasite strains, there was consistent agreement in RESA-IFA titers, regardless of the geographic origin of the parasites. The exception to this statement, and thus the evidence for strain variation to RESA-IFA, was only consistently seen in adult sera from the hyperendemic but unstable Jonglei Province, Sudan, mesoendemic Ecuador, and pediatric sera from Sudan. A possible explanation for this situation is that there exists only a limited number of variants for this antigen; thus, by the time most individuals living in malarious areas reach adulthood they have been exposed to all, or most, of the different variants and have antibodies to them. This situation would be similar to the one so elegantly demonstrated by Marsh and Howard (1986) for other falciparum parasite-infected erythrocyte surface antigens. In that study, antibodies from malarious children in The Gambia reacted with agglutination-inducing antigens on their own infected erythrocytes, but did not react with similar antigens on infected erythrocytes from other malarious children. Conversely, adult sera collected in the same area reacted consistently with most

of the infected erythrocytes derived from the children. The explanation given for these results proposed that adults had antibodies of multiple specificities, whereas the children had a limited antibody spectrum, restricted by their experience with the disease. It is possible that RESA antigen contains epitopes having variable immunogenicities, and thus children being less experienced with malaria than the adults might respond to the most dominant epitopes while adults eventually develop reactivity to all the epitopes. Our results could support both hypotheses for RESA. Sera containing high concentrations of RESA antibodies revealed little or no antigenic diversity, in support of the observations of others (Perlmann et al., 1984; Wahlin et al., 1984). However, because we examined many sera having low titers to RESA (Ecuador, Sudanese children), or from extremely remote regions (Jonglei, Sudan), we were able to detect variations in endpoint titers with different parasite strains. Unlike the report of Wahlgren et al. (1986), whose sera were collected in Liberia, we found no age-related increase in RESA-IFA titers in the pediatric sera we examined. There exist several possible explanations for these differences. Although our pediatric sera were collected from a hyperendemic area of Sudan, there exist, nonetheless, distinct seasons where malaria transmission is significantly greater than at other times. Thus, although the age span covered by the pediatric samples in both studies is similar, the actual exposure to malaria is undoubtedly higher in Liberia than in this part of Sudan. Furthermore, because of the unstable nature of transmission, some of our sera could represent acute, rather than chronic, malaria exposure. Altogether, our observations support the suggestion by Perlmann et al. (1984) that their failure to demonstrate antigenic diversity may have been due to the fact that their reference sera were too polyspecific, and their number of parasite strains limited.

It has recently been reported that the 155-kDa molecule known as RESA contains 2 repetitive amino acid sequences located near its 3′ end (Cowman et al., 1984; Collins et al., 1986). Although antibodies to both of these repeat sequences react positively in the RESA-IFA test, only antibody to 1 region was associated with protection when *Aotus* monkeys were immunized with *Escherichia coli*-derived fused polypeptides of the RESA molecule. Furthermore, it was reported that naturally acquired antibody to RESA in human serum was more often associated with the repetitive sequence that was not associated with protection (Collins et al., 1986). If this is true, the antigenic variation seen in this report may be due to differences in antibody titers between these 2 different epitopes, both of which are RESA-IFA reactive, but which likely have different degrees of immunogenicity, or may indeed by antigenically variable.

Regarding the reported observations that RESA-IFA titers were strongly correlated with inhibition of merozoite invasion *in vitro* (Perlmann et al., 1984; Wahlin et al., 1984), we report further observations on this matter. Whereas it is true that we never examined any sera having elevated titers of RESA-IFA antibody that were not likewise highly inhibitory to merozoite invasion, the inverse relationship was not true. Several sera having low RESA-IFA titers were nonetheless significantly inhibitory to merozoite invasion. Plausible explanations for these observations are neither surprising nor unexpected. As interesting as this parasite–erythrocyte surface antigen is, it is only one of several that may play a relevant role in protective immunity to blood-stage infections in falciparum malaria (Heidrich, 1986). Thus, it is reasonable to assume that by the time an individual has been exposed to malaria sufficiently to develop an elevated RESA-IFA titer, such a person could also have significant serum concentrations of other important merozoite-inhibiting antibodies. Furthermore, the molecule responsible for this RESA reaction actually contains 3 different immunologically relevant epitopes. From the 3′ end of the RESA molecule there are 2 repetitive sequences that respond to antibodies associated with erythrocyte surface immunofluorescence, only one of which induces protective immunity. Perhaps the most immunologically important epitope is located on the 5′ end and this antigen is nonreactive in the RESA-IFA test (Collins et al., 1986). Thus, it is possible that an individual could have significant antibody to the 5′ repetitive sequence, which is highly inhibitory to merozoite invasion, and low RESA-IFA titers.

In conclusion, our results have substantiated, in part, the observations that sera having high concentrations of RESA-IFA antibody show only occasional and minor evidence of antigenic diversity for those regions of the molecule responsible for the surface fluorescence. On the other hand, sera having low concentrations of RESA-IFA antibody may display variable endpoint titers, or no evidence of antibody, depending upon

the strains of parasites used in the assay. Moreover, although sera having high RESA-IFA antibody titers were uniformly inhibitory to merozoite invasion *in vitro*, the inverse relationship was not predictable. Several sera having low RESA-IFA titers were nonetheless highly inhibitory to merozoite invasion. Although these observations do not negate the value of the RESA-IFA test as an epidemiologic tool, they do help to define its useful parameters.

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ANTIGENS OF *CRYPTOSPORIDIUM* SPOROZOITES RECOGNIZED BY IMMUNE SERA OF INFECTED ANIMALS AND HUMANS

Jan R. Mead, Michael J. Arrowood, and Charles R. Sterling

Departments of Microbiology and Immunology, and Veterinary Science, University of Arizona, Tucson, Arizona 85721

ABSTRACT: The humoral response of humans, calves, and horses to *Cryptosporidium* sporozoite antigens was evaluated using a western blot technique. Sera from calves, humans, and horses were obtained at various times following the detection of infection. Sera were reacted with detergent-solubilized, sodium dodecyl sulfate polyacrylamide gel-electrophoresed (SDS-PAGE) sporozoite antigens. The number of antigens recognized by immune sera from humans and animals increased with time postinfection. A 20-kDa antigen appears to be a major sporozoite surface determinant labeled via membrane protein biotinylation and recognized by mouse monoclonal antibodies using indirect immunofluorescence and western blotting. Detectable recognition of the 20-kDa band occurred in 3-wk postinfection (PI) sera from all species tested. Reactivity to the 20-kDa band diminished significantly in sera 5 mo PI or longer from infected humans with no known recurrence of cryptosporidial diarrhea. In contrast, 12-mo PI sera from an individual constantly exposed to oocysts under working conditions was as strongly reactive as the 3-wk convalescent sera. Serum reactivity to the 20-kDa antigen appears to be a good indicator of exposure to *Cryptosporidium*.

Cryptosporidium parvum, an intestinal protozoan parasite primarily inhabiting the brush border of enterocytes, has been found to be a significant cause of diarrheal disease in man (Fayer and Ungar, 1986) and several animal species including calves (Pohlenz et al., 1978), chickens (Dhillon et al., 1981), horses (Snyder et al., 1978), and lambs (Barker and Carbonell, 1974). Infected immunocompetent humans usually have a short-term illness accompanied by watery diarrhea, whereas immunocompromised individuals may experience long-term diarrheal illness. In humans, protracted diarrheal disease caused by *Cryptosporidium* has been reported in AIDS patients (Centers for Disease Control, 1982), individuals undergoing immunosuppressive treatment (Meisel et al., 1976; Weisburger et al., 1979; Holley and Thiers, 1986), and hypogammaglobulinemic patients (Lasser et al., 1979; Sloper et al.,1982).

Serum antibodies to *Cryptosporidium* have been detected in both immunocompetent and immunocompromised individuals (Campbell and Current, 1983; Ungar et al., 1986) and in several animal species (Tzipori and Campbell, 1981). Random serologic-based studies in humans and animals have suggested that infection with this organism is common sometime during life (Tzipori and Campbell, 1981; Ungar et al., 1986). Immunocompetent individuals show typical patterns of IgM and IgG production following infection. The latter may diminish within a few months, or persist for a year or more (Campbell and Current, 1983; Ungar et al., 1986). Elevated IgA and IgE responses also have been noted (Casemore, 1987). A 23-kDa oocyst antigen is frequently recognized by sera of most infected humans (Ungar and Nash, 1986). Interestingly, cryptosporidial infections were less prevalent among breast-fed infants in Costa Rica than among nonbreast-fed infants, suggesting a possible protective role for mother's milk (Mata et al., 1984). In contrast, no apparent effect on the course of cryptosporidial infections was observed in calves fed colostrum containing anticryptosporidial antibodies (Current, 1986).

The present work was initiated to study the comparative humoral responses of infected humans, calves, and horses to sporozoite antigens of *Cryptosporidium*. The sequential appearance, persistence, and diminution of antigen-specific antibodies in sera was compared using western blotting techniques. Sporozoite antigens were further characterized using monoclonal antibodies and biotinylation of surface components.

MATERIALS AND METHODS

Oocyst production and purification

Cryptosporidium oocysts originally isolated from Holstein calves and obtained from Dr. Harley Moon (NADC, Ames, Iowa) were used to infect 2–5-day-old Holstein calves (10^8/animal). Following the onset of oocyst shedding, feces were collected daily, mixed with an equal volume of 5% potassium dichromate ($K_2Cr_2O_7$), and stored at 4 C. Feces were sieved sequentially through stainless steel screens with a final mesh size of 230 (63 μm porosity).

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Oocysts were purified from feces using discontinuous sucrose and isopycnic Percoll (Pharmacia, Piscataway, New Jersey) gradient centrifugation techniques (Arrowood and Sterling, 1987). In brief, sieved feces were centrifuged over 2 sequential discontinuous sucrose gradients prepared using 0.025 M phosphate-buffered saline (PBS, pH 7.2) and 1% Tween 80. Each gradient tube was composed of 2 10-ml lower sucrose layers (1.064 g/ml and 1.103 g/ml) and a 5-ml upper layer of sieved feces. The tubes were centrifuged at 1,500 *g* for 30 min and oocysts were recovered from the interface of the sucrose layers and washed with PBS at 1,500 *g* (10 min × 3). A 1-ml aliquot of sucrose gradient-recovered oocysts in PBS was layered over 9 ml of Percoll in a high-speed centriuge tube and centrifuged at 22,000 *g* for 30 min at room temperature. Oocysts were recovered from the centrally located band in the Percoll gradient.

Sporozoite preparation

Percoll gradient-recovered oocysts were washed with PBS at 1,500 *g* (10 min × 3) and resuspended to 20 ml PBS at a concentration of 1–2 × 10^8/ml. The oocyst suspension was mixed with an equal volume of excysting solution (0.5% trypsin, 1.5% sodium taurocholate in PBS) and incubated 40–60 min in a 37 C water bath (Fayer and Leek, 1984). The sporozoite mixture was washed with Alsever's solution and resuspended to a volume of 4 ml. A 1-ml aliquot of excysted sporozoites was layered over 9 ml of the Percoll solution and centrifuged as described for oocysts. The centrally located sporozoite band recovered from the Percoll gradients was washed with PBS and counted using a hemacytometer. Approximately 2–3 × 10^9 sporozoites were pelleted and resuspended with 1 ml of 150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% sodium azide (NET) buffer, pH 7.4, containing 0.5% of the nonionic detergent Nonidet P-40, and 1 mM each of the enzyme inhibitors *N*-alfa-tosyl-L-lysylchloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, Missouri) (Zingales and Colli, 1984). After incubating at room temperature for 10 min the suspension was centrifuged at 20,000 *g* for 2 min. The supernatant-solubilized membrane preparation was decanted and stored at 4 C until used.

Serum samples

Serial serum samples were collected from experimentally infected calves (used for oocyst production) prior to infection and at 10 days, 3 wk, 10 wk, 16 wk, 20 wk, and 28 wk postinfection (PI). Additional serum samples were obtained from naturally infected calves. Other control calf sera were obtained from gnotobiotic calves (courtesy of Dr. Harley Moon).

Serial serum samples were also obtained from naturally infected humans and horses at various times following the onset of clinical symptoms and detection of oocysts in stools. Sera from individuals returning from Mexico were similarly collected and shipped to our laboratory through the coordination of William Keene, Department of Health Services, State of California, and Stephen Waterman, Department of Health Services, County of Los Angeles. These sera were taken approximately 1 mo after clinical signs of cryptosporidiosis developed in patients returning to the United States. Postconvalescent sera from 4 of these individuals were obtained 9 mo after the first serum samples were collected. Because sera were collected at various time periods and quantities of sera were limited, western blots of some sera were performed on separate electrophoretic runs. Control human sera were obtained from individuals with no known exposure or diarrheal illness attributable to *Cryptosporidium*. Positive horse sera were supplied to our laboratory by Thomas Klei, Louisiana State University. These horses had diarrheal symptoms attributed to *Cryptosporidium* infection. Control horse sera were obtained from an 8-yr-old Arizona Morgan mare with no known history of diarrheal illness. All sera were stored at −20 C.

Monoclonal antibody production

Adult BALB/c mice were immunized with approximately 1 × 10^6 oocysts and sporozoites (OS). Immunizations were carried out on days 0 (OS + Freund's complete adjuvant delivered intramuscularly), 14 (OS + Freund's incomplete adjuvant delivered intraperitoneally), and 28 (OS delivered intravenously) (Sterling and Arrowood, 1986). On day 32 the spleens were removed and fused with P3/X63/Ag8.653 mouse myeloma cells employing polyethylene glycol 4000 (E. Merck, Darmstadt, West Germany). The resulting hybridoma cells were grown in hypoxanthine, aminopterine, thymidine (HAT)-selective RPMI-1640 supplemented with 15% fetal calf serum in 24-well culture plates (Fazekas et al., 1980). Supernatants were screened for specific antibodies using an indirect immunofluorescent assay against air-dried *Cryptosporidium* sporozoites and oocysts (Sterling and Arrowood, 1986).

Hybridomas secreting antibodies to sporozoite determinants were cloned by limiting dilution and subclassed by indirect immunofluorescent assay using isotype-specific biotinylated anti-mouse immunoglobulin antisera (Zymed Laboratories Inc., South San Francisco, California). Culture supernatants from serially passaged hybridomas were stored at 4 C until used.

Polyacrylamide gel electrophoresis (PAGE)

Gradient gels of 10–20% polyacrylamide or standard 10% polyacrylamide gels were used with a discontinuous buffer (Laemmli, 1970). A 250-μl sporozoite membrane sample (approximaely 1.05 mg/ml) was diluted with an equal volume of sample buffer (100 mM phosphate buffer, 1% sodium dodecyl sulfate [SDS], 140 mM 2-mercaptoethanol, 0.015% bromophenol blue, 6.0 M urea, and 10% glycerol) and boiled 4 min. Electrophoresis was performed at a constant 100 mA at 18 C. Prestained high and low molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Maryland) were incorporated into each electrophoretic run. Gels not used for western blotting were fixed and silver stained using the Gel Code silver stain kit (Pierce Chemicals, Rockford, Illinois).

Western blotting

Following SDS-PAGE, proteins were transferred to nitrocellulose (Towbin et al., 1979) using a Bio-Rad Trans-Blot cell (Bio-Rad Laboratories, Richmond, California). Electrophoretic transfer at 4 C was achieved by employing a constant 30 volts overnight followed by 60 volts for 2 hr. The efficiency of protein transfer was determined by silver staining the polyacrylamide gel after transfer as well as by staining nitrocellulose

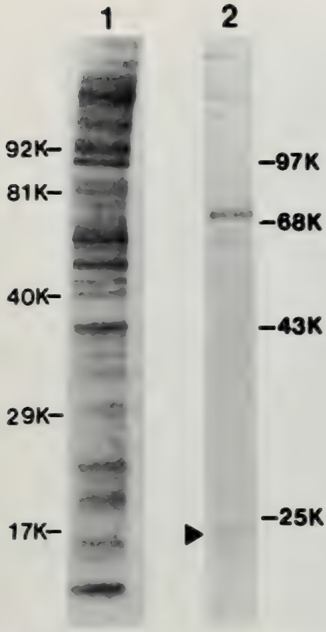

FIGURE 1. Silver-stained 10–20% gradient gel SDS-PAGE of a sporozoite membrane preparation (lane 1). Lane 2 shows a western blot of biotinylated sporozoite membrane antigens. Arrow denotes 20-kDa antigen.

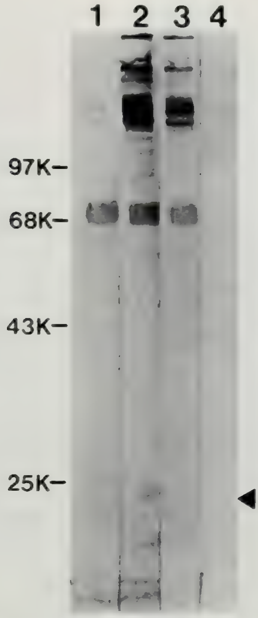

FIGURE 2. Western blot of an individual infected with *Cryptosporidium*. Preinfection, 3-wk, and 10-mo PI sera are shown in lanes 1–3, respectively. Lane 4 shows the western blot of a control human serum.

strips with amido black. Following transfer, nitrocellulose strips were blocked for 30 min in a solution of 1.0% powdered goat milk dissolved in 0.05 M Tris-buffered saline (TBS), pH 7.5 (Johnson et al., 1984). The blocked strips were incubated in diluted sera (1:25 in TBS) for 1 hr, washed with TBS, and incubated for 1 hr with either biotinylated anti-horse IgG (Vector Laboratories Inc., Burlingame, California), biotinylated anti-human IgG + IgM (Bethesda Research Laboratories), or biotinylated anti-bovine IgG (Kirkegaarde and Perry Laboratories, Gaithersburg, Maryland) diluted 1:1,000 in TBS. The strips were then washed with TBS and reacted for 1 hr with streptavidin–horseradish peroxidase (Bethesda Research Laboratories) diluted 1:1,000 in TBS. After a final TBS wash, color development was accomplished using 0.05% 4-chloro-1-naphthol and 0.015% H_2O_2 (Kirkegaarde and Perry Laboratories). Transfer conditions and reagent dilutions were identical in all blotting experiments.

Biotinylation of sporozoite surface proteins

Sporozoites were purified and suspended in PBS at a concentration of 5×10^7/ml. Aliquots (2 ml) were dispensed to each of 2 tubes. Tube A was centrifuged and the sporozoite pellet solubilized in NET buffer. Tube B received 40 μl of a 10-mg/ml solution of sulfo-NHS-biotin (Pierce Chemicals) dissolved in dimethyl sulfoxide (Hurley et al., 1985). The tube was incubated at room temperature for 10 min with agitation. After centrifugation, the pellet of labeled sporozoites was solubilized as described above. The supernatant detergent fractions were electrophoresed, transferred to nitrocellulose, blocked, incubated with streptavidin–horseradish peroxidase, and reacted with 4-chloro-1-naphthol and H_2O_2 as described previously.

RESULTS

Polypeptides of *Cryptosporidium* sporozoites

Silver staining of SDS-PAGE gels (Fig. 1, lane 1) detected a total of 46 bands ranging in molecular weight from approximately 300 kDa to 3 kDa. The majority of these bands were transferred to nitrocellulose as demonstrated by amido black staining (results not shown). Western blots of biotinylated sporozoite surface proteins identified a subset of the bands observed in the silver stain (Fig. 1, lane 2). All of the biotinylated proteins correlated with antigens identified in western blots reacted with human and/or animal immune sera. Immune reactivity to one of these, a 20-kDa biotinylation product, was recognized by all immune sera tested.

Western blots—human sera

Western blots of human sera reacted against sporozoite antigens are shown in Figures 2–4.

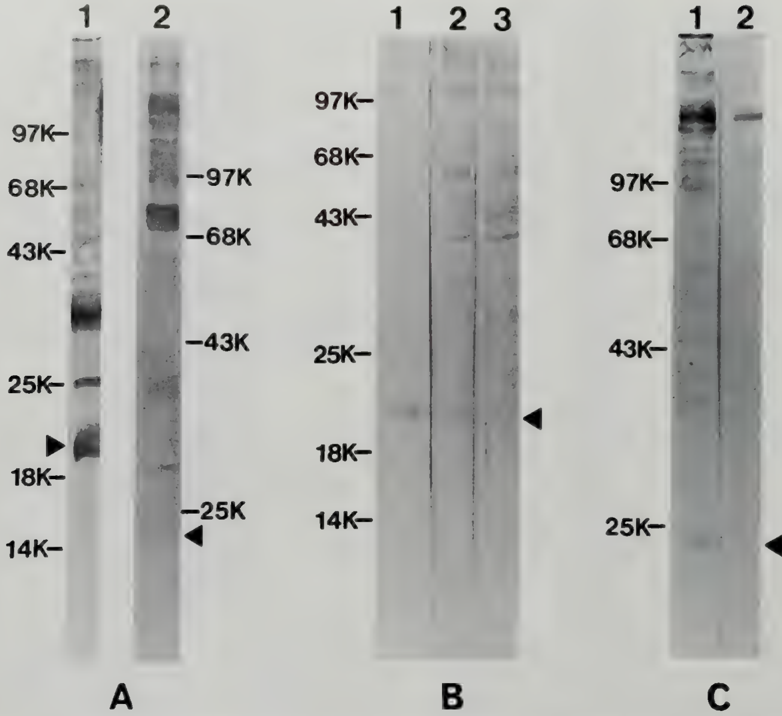

FIGURE 3. Western blot analysis of sporozoite antigens reacted with human sera from individuals infected with *Cryptosporidium*. Panel A, lanes 1 and 2 show 3-wk and 1-yr PI sera, respectively, from individual B. Panel B shows blots from individual C using 10-day, 5-mo, and 9-mo PI sera (lanes 1, 2, and 3, respectively). Panel C, lanes 1 and 2 show serum reactivity for individual D taken 4 wk and 10-mo PI, respectively.

Individual A contracted cryptosporidiosis which was detected in our laboratory by direct and indirect immunofluorescent assays for oocysts in submitted stools. The 3-wk PI serum (Fig. 2, lane 2) of this individual reacted with a 20-kDa antigen not seen in either the preinfection serum (Fig. 2, lane 1) or the 10-mo PI serum (Fig. 2, lane 3). A blot reacted with a control serum is shown in Figure 2, lane 4. Several high molecular weight bands were evident in these blots; 2 apparent in the preinfection serum of individual A (Fig. 2, lane 1). Several high molecular weight bands were virtually indistinguishable between the 3-wk (lane 2) and 10-mo (lane 3) PI sera.

Serum reactivity of individual B is shown in Figure 3, panel A. The strong 3-wk PI serum reactivity to the 20-kDa antigen (lane 1) was not observed in a separate analysis of the 12-mo followup serum (lane 2), whereas several higher molecular weight bands were apparent.

Serum reactivity of individual C, who contracted cryptosporidiosis while traveling to Mexico, is shown in Figure 3, panel B. *Cryptosporidium* oocysts were detected in the patient's stool during symptoms of acute diarrhea and up to 7 days following the cessation of symptoms (Sterling et al., 1986). The 20-kDa antigen was apparent in blots using 10-day PI serum (lane 1), less apparent using 5-mo PI serum (lane 2), and absent using 9-mo PI serum (lane 3).

Western blots were also performed on convalescent sera from a group of tourists returning from a trip to Puerto Vallarta, Mexico, who were involved in a possible outbreak of cryptosporidiosis. Oocysts were identified in the stools of 2 individuals and others reported symptoms typical of cryptosporidiosis. Serum reactivity of individual D who was among this group is shown in Figure 3, panel C. The 4-wk PI serum reacted with the 20-kDa antigen (lane 1). The followup 10-mo serum (lane 2) showed no reactivity toward this antigen. Western blots of 23 additional individuals from this symptomatic group showed reactivity to the 20-kDa antigen. Two additional

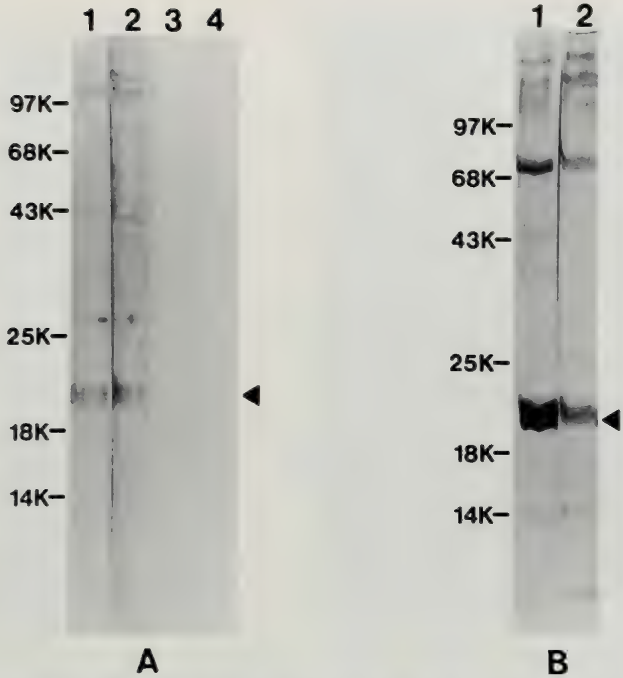

FIGURE 4. Panel A, lanes 1 and 2 show serum reactivities of individual E at 3 wk and 1 yr PI, respectively. Control sera are shown in lanes 3 and 4. Panel B, lanes 1 and 2 show 4-wk PI sera from 2 individuals who had symptoms of cryptosporidiosis after returning from a trip to Puerto Vallarta, Mexico.

serum reactivities are illustrated in blots presented in Figure 4, panel B, lanes 1 and 2.

The serum reactivity of individual E, a person who had continued exposure to *Cryptosporidium*-infected calves is shown in Figure 4, panel A. This individual contracted cryptosporidiosis while working with *Cryptosporidium*-infected calves. Sera taken from this individual at 3 wk (panel A, lane 1) and 1 yr PI (panel A, lane 2) recognized similar antigens. No decrease in intensity of any of the bands, including the 20-kDa band, was observed.

Western blots—bovine sera

Western blots using sera from calf A (Fig. 5) showed increasing band number and intensity with time. Sera showed reactivity to several bands of high molecular weight and the 20-kDa band. The 20-kDa band observed using 3-wk PI serum (panel A, lane 4) became more prominent at 10 wk PI (panel A, lane 5). In a subsequent set of blots, reactivity toward this band was strong using 16-wk PI serum (panel B, lane 1), whereas it was absent using 20- and 28-wk PI sera (panel B, lanes 2 and 3, respectively). Gnotobiotic control serum (panel A, lane 1) and serum taken 3 days PI (results not shown) demonstrated negligible blot reactivity to sporozoite antigens. Sera from naturally infected calves and other experimentally infected calves displayed blot reactivities similar to those described above for the infected calf.

Western blots—equine sera

Serum samples taken 3 wk PI from horse A (Fig. 6, lane 1) and horse B (Fig. 6, lane 2) recognized several antigenic bands including the 20-kDa band. The serum of horse C (taken 4 mo PI) reacted with high molecular weight bands like the sera of horse A, but reactivity to the 20-kDa band was not evident (lane 3). One band of high molecular weight was barely visible using a control horse serum (lane 4).

Western blots and immunofluorescence using monoclonal antibodies

Monoclonal antibodies C3B4, C6B6, C1D3, and C8C5 all show recognition of the same 20-

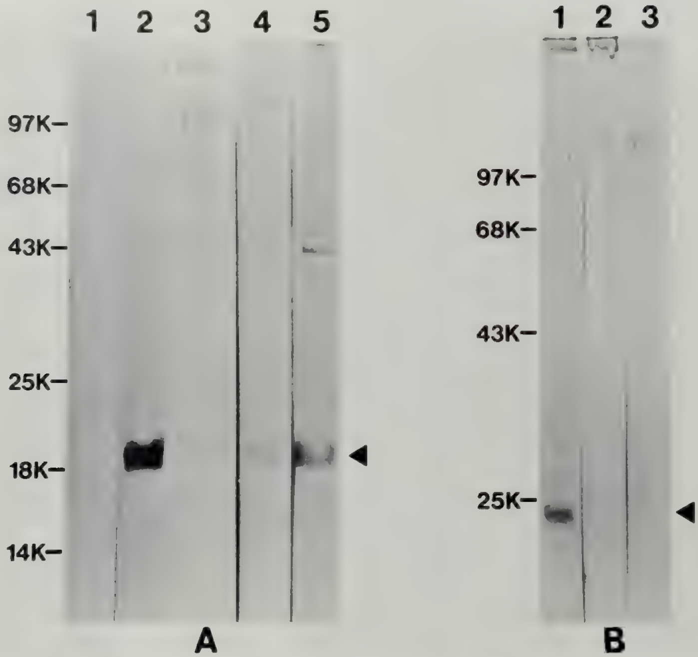

FIGURE 5. Western blots containing sporozoite antigens reacted with sera taken from an experimentally infected calf at 10 days, 3 wk, 10 wk (panel A, lanes 3–5, respectively), and 16 wk, 20 wk, 28 wk PI (panel B, lanes 1–3). Panel A, lane 1 shows negligible blot reactivity of gnotobiotic calf sera. Monoclonal C6B6 shows strong reactivity to the 20-kDa antigen (panel A, lane 2).

kDa antigen band (Fig. 5, lane 2, C6B6 shown) in western blots. The first 3 hybridomas secrete IgG1 subclass monoclonals whereas the latter secretes an IgG3 monoclonal. This band coincides with the 20-kDa band identified by immune sera. The typical indirect immunofluorescent surface labeling of sporozoites using monoclonal antibodies C3B4, C6B6, C1D3, and C8C5 (C6B6 shown) is shown in Figure 7.

DISCUSSION

The reactivity of sera from humans, calves, and horses to *Cryptosporidium* sporozoite antigens was investigated using a western blot technique. Sera taken from experimentally infected calves and naturally infected horses and humans demonstrated recognition of specific sporozoite membrane antigens. High molecular weight antigens (96–200 kDa) also were recognized by some of the control sera.

Convalescent sera from *Cryptosporidium*-infected calves, horses, and humans all recognized a 20-kDa sporozoite antigen in western blots. Response to this 20-kDa antigen appeared and peaked during convalescence but diminished during postconvalescence. Responses to some high molecular weight antigens persisted for more than a year following infection. In contrast, human sera from one individual reacted strongly to the 20-kDa antigen up to a year after his initial infection. No diarrheic episodes attributable to *Cryptosporidium* occurred during this period and periodic stool examinations demonstrated no detectable oocyst shedding. This individual had continuous potential for reexposure to oocysts during routine handling of experimentally infected animals. Conceivably, continued antigenic stimulation maintained the serum response to the 20-kDa antigen.

A 23-kDa antigen of sonicated oocysts recognized by immune sera of patients in a previous study (Ungar and Nash, 1986) probably corresponds to the 20-kDa antigen of purified sporozoites demonstrated by the results of the current investigation. Molecular weight differences

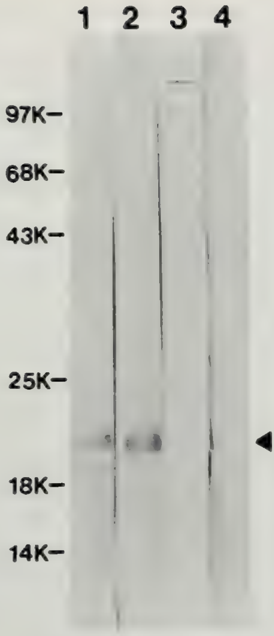

FIGURE 6. Western blot of sporozoite antigens reacted with equine sera from horse A at 3 wk PI (lane 1), from horse B at 3 wk PI (lane 2), horse C at 4 mo PI (lane 3), and an uninfected control horse (lane 4).

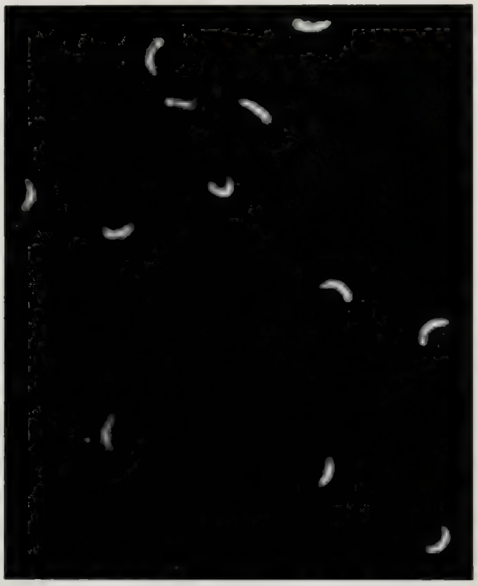

FIGURE 7. Indirect immunofluorescent appearance of monoclonal C6B6 reacted with air-dried sporozoites showing typical surface labeling. ×3,600.

are probably due to differing gradient gel applications. Evidence to confirm the sporozoite membrane origin of this antigen includes sporozoite monoclonal antibody reactivity in western blots, immunofluorescent patterns, and biotinylation of membrane proteins.

Interestingly, Lazo et al. (1985) did not observe reactivity to antigens below 60 kDa when testing 40-day PI sera of a *Cryptosporidium*-infected calf. It is not clear why their results differ from those of the present study.

The observed similarities among western blots of immune sera taken from calves, horses, and humans suggests that several antigens, and especially the 20-kDa antigen, are probably conserved among the infecting cryptosporidia. Minor band variations between blots appear to be as great within species (see human blots) as between species (e.g., horse vs. calf). Because the sporozoites used in the western blots were originally derived from a single calf isolate, variation in blotting patterns implies a heterogeneity in humoral responsiveness. Alternatively, antigenic differences (strain variation) might exist among the infecting cryptosporidia.

Cryptosporidium differs from other coccidian parasites presumably by recycling sporozoites through the generation of thin-walled oocysts (Current and Long, 1983). It is therefore not surprising that the host mounts a humoral response to this life cycle stage. Antibody cross-reactivity between sporozoites and merozoites may also exist should these stages share antigenic determinants. In this regard, the monoclonal C6B6, which binds the 20-kDa surface antigen of sporozoites, reacts with merozoites recovered from the ileum of infected mice via indirect immunofluorescence (data not shown). Western blots of merozoite membrane antigens have not yet been performed, thus limiting the characterization of this cross-reactive antigen.

The western blot technique described in the present study has confirmed the presence of a 20-kDa sporozoite surface antigen recognized by immune sera of humans, which is also recognized by the sera of different animals. Additional characterization of this antigen as a surface protein is supplied by the biotinylation and immunofluorescence data. Reactivity to this antigen, in the absence of reexposure, appears to decline following convalescence. Serum recognition of this antigen, therefore, probably correlates with recent exposure to *Cryptosporidium*.

ACKNOWLEDGMENTS

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BOOK REVIEW . . .

The Proceedings of the Heartworm Symposium '86, G. F. Otto (ed.). American Heartworm Society, 21–23 March 1986. 223 p.

The Proceedings of the Heartworm Symposium '86, edited by Gilbert F. Otto, follows the practice of the American Heartworm Society to publish authoritative surveys of recent developments in research on dirofilariasis. The material contained in this text was presented during the American Heartworm Society Symposium held in New Orleans, Louisiana during March 1986, reportedly the most comprehensive symposium on the topic held to date.

The text begins with an extensive review of the use of ivermectin as a new approach to prevention as well as treatment of dirofilariasis. Following a brief discussion of the mode of action of the drug, the efficacy and safety of ivermectin against canine dirofilariasis is compared to that of diethylcarbamazine. This section, which should be of particular interest to veterinary practitioners, has been more than doubled in length and content since the previous "Proceedings" of 1983. Further, the "Recommended Procedures for the Treatment and Prevention of Heartworm Disease" have been revised and included in this text.

Seven articles address the development of new diagnostic tests for dirofilariasis which, because they detect parasite antigens, promise to be more accurate than conventional serology. The new tests for antigen may also prove to be valuable in monitoring the success or failure of treatment with adulticides.

Feline dirofilariasis appears to be increasing and the "Proceedings" more than adequately cover this problem. The excellent article by Ray Dillion on feline heartworm disease presented in the 1983 "Proceedings" has been updated and lengthened in the 1986 text and three comprehensive papers addressing the incidence, preferred methods of diagnosis, and treatment of parasitized cats have been included.

The book contains numerous excellent tables and figures which presents considerable information of value to both the researcher and the veterinary practitioner. Reproduction of radiographs and photographs is reasonably good. Also included are transcripts of eight discussion sessions which were held following the presentations of groups of related papers. The editor rightly states that many of the comments and questions are not identified and that little or no attempt was made to document statements. However, the inclusion of the discussion sessions in the text is valuable in that the reader is provided with insights into problems of the prevention, diagnosis, treatment, and management of heartworm disease which are usually reserved for discussions over coffee by those able to attend the meeting. A bibliography which supplements those published in earlier "Proceedings," is provided.

In summary, this book should appear on the bookshelves, not only of veterinarians who confront the problems of heartworm disease on a daily basis, but also of researchers who value the availability of a single, up-to-date source of information on a wide variety of topics concerning dirofilariasis.

A. C. Vickery, College of Public Health, University of South Florida, Tampa, Florida 33612.

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B-LYMPHOCYTE RESPONSES IN THE LARGE INTESTINE AND MESENTERIC LYMPH NODES OF MICE INFECTED WITH *EIMERIA FALCIFORMIS* (APICOMPLEXA)

Patricia V. Nash and C. A. Speer
Veterinary Research Laboratory, Montana State University, Bozeman, Montana 59717

ABSTRACT: B-cell responses of 3 immunoglobulin isotypes (IgA, IgG, and IgM) were investigated in the large intestine and mesenteric lymph nodes (MLN) of naive or immune mice after inoculation of oocysts of *Eimeria falciformis*. Primary and anamnestic IgA and IgG lymphocyte responses to *E. falciformis* occurred in the large intestine of nonimmune and immune mice, respectively. IgA-containing lymphocytes (IgAc) were the largest population of responding B cells in the large intestine. In infected mice, IgAc accumulated in the apical portion of the lamina propria, whereas IgG-containing lymphocytes (IgGc) were more numerous at the base of the lamina propria. No significant increase in the number of IgM-containing lymphocytes (IgMc) was observed in the lamina propria of the large intestine. Primary but no anamnestic B-cell responses occurred in the MLN, and immune mice actually had reduced numbers of IgAc and IgGc in the MLN when compared with naive mice. IgGc were the largest population of responding B cells in the MLN. Thus, IgAc appear to accumulate preferentially at the site of parasite development, whereas IgGc are primarily localized deeper in the lamina propria of the large intestine and in the draining lymph nodes of mice infected with *E. falciformis*.

Local rather than systemic immune responses are probably more important in acquired resistance to intestinal coccidia, but few studies have been done on the mucosal immune response to these parasites. Both antibody- and cell-mediated responses appear to be involved in protective immunity (Orlans and Rose, 1972; Joyner and Norton, 1974; Mesfin and Bellamy, 1979a; Rose and Hesketh, 1979; Lillehoj, 1987), although the role of each system is unclear. Substantial amounts of parasite-specific antibodies are present in the serum of animals infected with coccidia (Andersen et al., 1965; Mesfin and Bellamy, 1980; Whitmire and Speer, 1986). Such antibodies, however, probably have limited access to parasite antigens in the intestinal mucosa. Chickens appear to develop both primary and anamnestic mucosal IgA responses to *Eimeria tenella* (Davis and Porter, 1979), and parasite-specific IgA and IgG have been detected in mucosal and fecal extracts from mice infected with *E. falciformis* (Douglass and Speer, 1985; Whitmire and Speer, 1986).

Eimeria falciformis infects the large intestine and cecum of mice. Severely infected mice develop lesions in the intestinal mucosa and may experience retardation of growth, morbidity, and mortality (Mesfin et al., 1978; Speer and Pollari, 1984). We report herein the distribution and kinetics of the IgA-, IgG-, and IgM-positive lymphocyte responses in the large intestine and mesenteric lymph nodes of immunized and naive mice challenged with oocysts of *E. falciformis*.

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MATERIALS AND METHODS

Mice

Twelve-wk-old, age-matched female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) were housed in a specific pathogen-free environment in the Montana State University animal facility. Control and infected mice were kept in separate rooms, and animals passing oocysts were placed in cages with wire-mesh bottoms to minimize retroinfections. Mice were checked for intestinal coccidia and helminths by sucrose fecal flotation prior to use in an experiment. Mice also tested negative for antibodies against *Mycoplasma* and various murine viruses (e.g., mouse adenovirus, Sendai virus, lymphocytic choriomeningitis, mouse hepatitis virus, minute virus, rheovirus 3, etc.).

Parasite

Mice were inoculated by gavage with approximately 10^3 sporulated oocysts of *E. falciformis* and placed in cages with wire-mesh floors. Feces were collected on 7, 8, and 9 days postinoculation (DPI). Oocysts were recovered by flotation in Sheather's sugar solution, sporulated by aeration in 2.5% aqueous $K_2Cr_2O_7$ for 3–5 days at 23 C, and stored in 2.5% aqueous $K_2Cr_2O_7$ at 4 C for no more than 2 mo. Immediately before use, oocysts were rinsed thoroughly with tap water.

Infection of mice

Ninety-six mice were divided into 3 groups of 32 animals, and given 1 of the following treatments: Group 1, control mice, remained untreated throughout the study; group 2, naive mice, each received 1 gavage inoculation of 10^3 oocysts on day 16 of the study; group 3, "immune" mice (Mesfin and Bellamy, 1979b), were each inoculated by gavage with 10^3 oocysts on day 0 and with 10^3 oocysts on day 16. Beginning at 16 days, 4 mice from each group were killed at 6 hr, and at 1,

3, 5, 7, 9, 11, and 13 days postchallenge (DPC) with oocysts. The experiment was repeated 6 mo later with naive and immune groups of mice.

Tissue collection and processing

Mice were killed by cervical dislocation and the large intestine and mesenteric lymph nodes (MLN) removed. The intestine was cut open longitudinally and the mucosal surface cleaned of fecal material with a cotton swab moistened with saline. Tissues were fixed for 3 hr in Bouin's fluid, dehydrated in ethanol, cleared in xylene, and infiltrated and embedded in low-temperature Surgipath embedding medium (Surgipath Medical Industries, Inc., Northbrook, Illinois). Tissue sections were fixed to glass slides with Histostik (Accurate Chemical and Scientific Corporation, Westbury, New York) and stained using the immunoperoxidase procedure for the identification of B lymphocytes containing cytoplasmic immunoglobulin. Tissue sections were counterstained with hematoxylin and mounted with permount (Fisher Scientific Company, Fairlawn, New Jersey).

Immunoperoxidase staining

An avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981) was used to stain tissue sections for the presence of lymphocytes containing IgA, IgG, or IgM. Rabbit anti-mouse IgA primary antiserum (Miles Scientific, Naperville, Illinois) was reconstituted, aliquoted, and frozen at −20 C. Sodium azide (0.1% final concentration) was added to heavy chain-specific rabbit anti-mouse IgG and anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc., Avondale, Pennsylvania). These antibodies were refrigerated at 4 C until used. Titrations of the primary and secondary antibodies were made to determine the appropriate concentrations for staining. The anti-IgA was diluted to a final concentration of 1:50, anti-IgG to 1:300, and anti-IgM to 1:800. A Vectastain Kit (Vector Laboratories, Burlingame, California), consisting of blocking serum, biotinylated anti-rabbit IgG, and the ABC reagent, was used to detect immunoglobulin-positive cells in the large intestine and MLN. The biotinylated secondary antibody was diluted to a final concentration of 1:200 for the IgA stain, and to 1:500 for the IgG and IgM stains. Diaminobenzidine tetrahydrochloride (Sigma Chemical Company, St. Louis, Missouri) and hydrogen peroxide were used to develop the brown precipitate on positive cells.

The staining procedure used was similar to that outlined in the Vectastain Kit except for the use of 0.5 M sodium chloride in the ABC reagent buffer to prevent nonspecific staining of mast cells.

Sections of 3 control tissues plus sections of experimental tissues were stained each day. One control consisted of sections of normal BALB/c mouse small intestine treated with diluted normal rabbit serum instead of the primary antibody. Sections of normal BALB/c mouse lung were used as a positive control for anti-IgA, and sections of an IgM-secreting mouse hybridoma (a solid tumor provided by Dr. Diane Brawner) were used as a negative control. Sections of the same IgM-secreting tumor were also used as a positive control for the anti-IgM antiserum, and an IgG-secreting solid tumor (MPC 11 OVA® plasma cell tumor line, American Type Culture Collection, Rockville, Maryland) was used as a negative control. The IgG-secreting tumor was used as a positive control for the anti-IgG antiserum, and the IgM-secreting tumor served as the negative control.

Statistics

The numbers of IgA-, IgG-, or IgM-positive cells (IgAc, IgGc, or IgMc, respectively) were determined by counting the number of positively stained cells in 10 randomly selected, contiguous high-power microscope fields (HPF) (0.13 mm^2 for the large intestine; 0.2 mm^2 for the MLN) of the large intestine and medulla of the MLN. Ig lymphocytes in sections from each of the 4 mice in a group were counted and the data expressed as the mean ± SD for each experiment. The large intestine was sectioned longitudinally and only those areas free of lymphoid nodules were examined for Ig-positive cells.

Numbers of IgAc, IgGc, or IgMc were square-root transformed and differences between groups in total number of Ig-positive cells were tested for significance ($P < 0.05$) by using single-factor analysis of variance and Duncan's multiple range test.

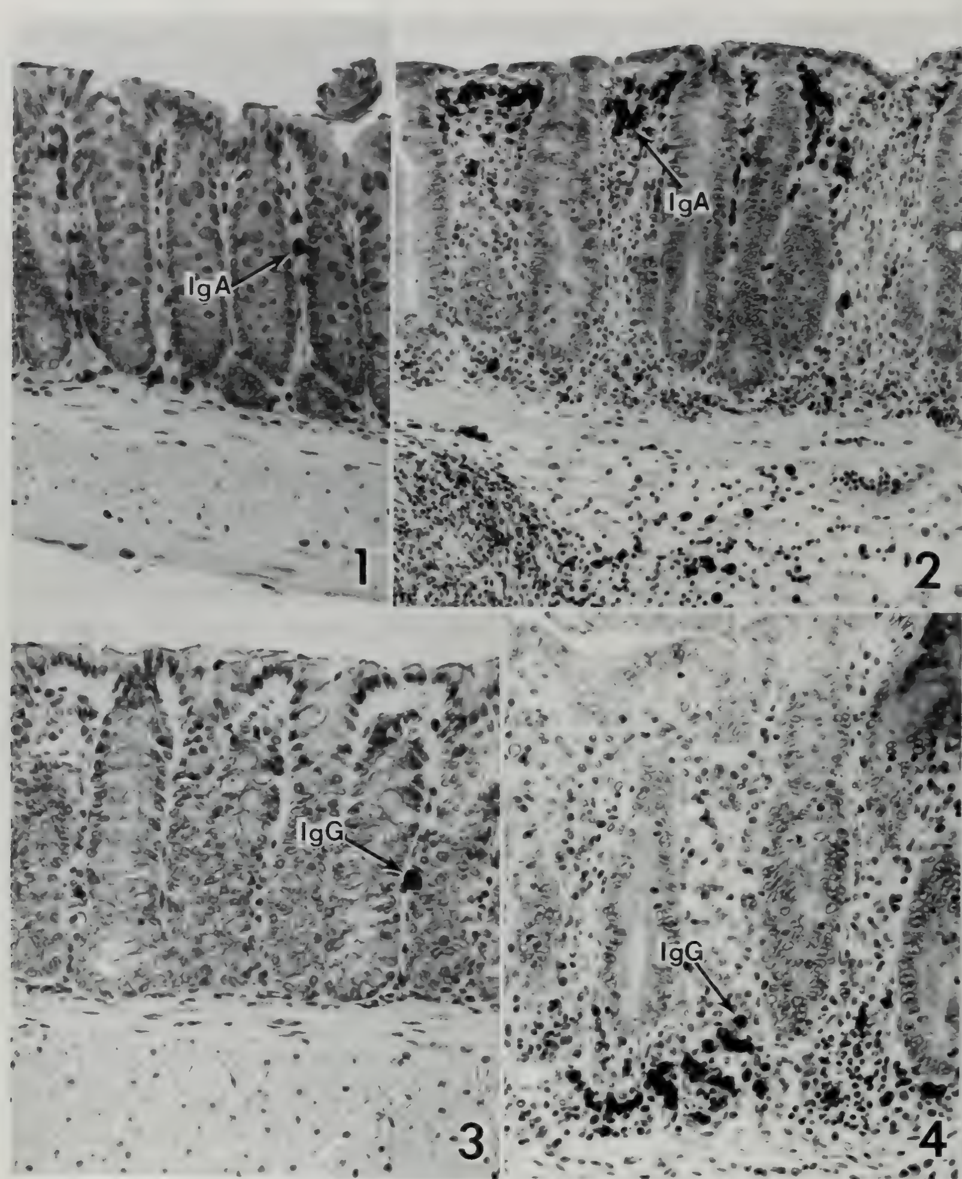
RESULTS

Lymphocyte distribution in the large intestine and mesenteric lymph nodes

In control (noninfected) mice, IgAc were located throughout the lamina propria in the large intestine (Fig. 1), and the IgAc density (67.2–117.0 cells/10 HPF) was higher than that of IgMc and IgGc (Fig. 3). In the intestinal mucosa of naive or immune mice inoculated with *E. falciformis,* IgAc were more heavily concentrated in the middle and especially the upper part of the lamina propria (Fig. 2). Some infiltration of IgAc also occurred in the basal lamina propria.

In control mice, IgGc were evenly distributed throughout the lamina propria of the large intestine, and the IgGc density (1.0–6.0 cells/10 HPF) was significantly less than that of IgAc. IgGc were concentrated at the base of the mucosa in the large intestine of naive or immune mice inoculated with *E. falciformis* (Fig. 4).

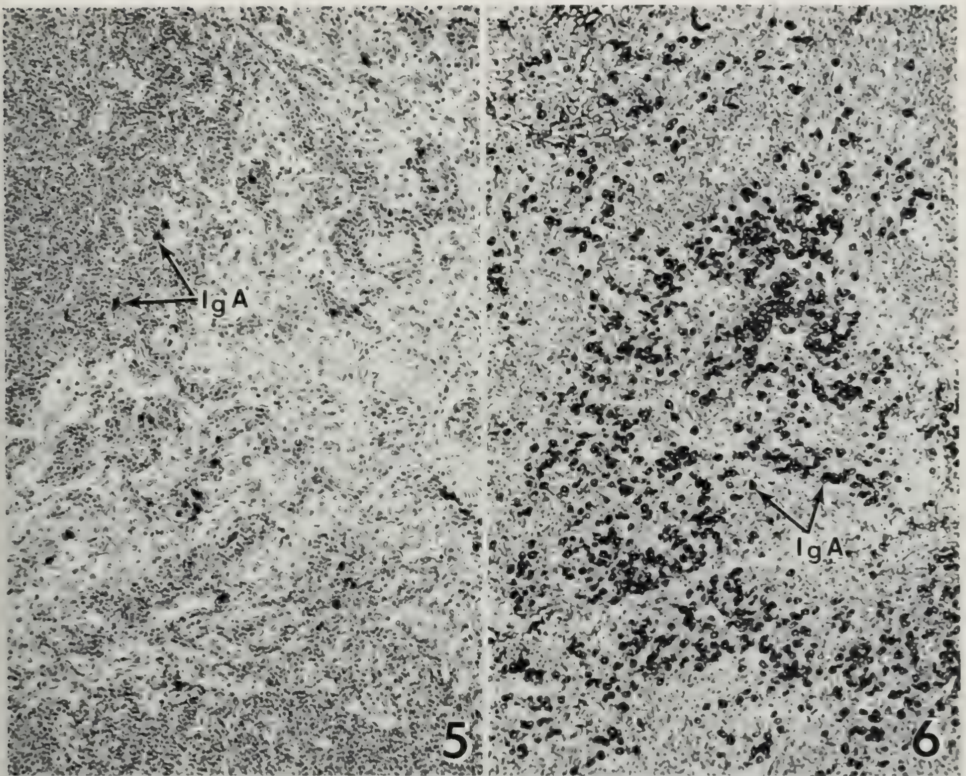
Relatively few (0–2.8 cells/10 HPF) and evenly distributed IgMc were present in the lamina propria of the large intestine in both control and infected mice. IgAc, IgGc, and IgMc were concentrated in the medulla of the MLN of both control and inoculated mice (Figs. 5, 6); few Ig-positive cells were seen within follicles. Occasional Ig-positive cells of all 3 classes were seen in the perifollicular areas of MLN in control mice. Challenged mice had significantly more Ig-positive cells surrounding the B-cell follicles than did control mice.



FIGURES 1–4. Photomicrographs of IgA-positive (Figs. 1, 2) and IgG-positive (Figs. 3, 4) lymphocytes in the lamina propria of the large intestine of control (uninfected) (Figs. 1, 3) and immune mice (Figs. 2, 4). **1.** Relatively few IgA-positive cells (IgA). ×160. **2.** Numerous IgA-positive cells (IgA) in the apical and central regions of the lamina propria; immune mouse 11 days postchallenge with *E. falciformis* oocysts. ×160. **3.** Relatively few IgG-positive cells (IgG). ×160. **4.** Numerous IgG-positive cells (IgG) at the base of the lamina propria; immune mouse 11 days postchallenge with *E. falciformis* oocysts. ×160.

IgA lymphocyte response

Figures 7 and 8 show t [illegible] the IgAc response in the large inte[illegible] ILN of immune and naive mice at 6 hr to 13 DPC with *E. falciformis*. The primary IgAc response in the large intestine of naive mice increased steadily and peaked at 11 and 13 DPI, during which the



FIGURES 5, 6. Photomicrographs of mesenteric lymph nodes of control (uninfected) (Fig. 5) and naive mice (Fig. 6) stained for IgA-positive lymphocytes. **5.** Few IgA-positive cells (IgA). ×100. **6.** Numerous IgA-positive cells (IgA) in medulla of mesenteric lymph node; naive mouse 11 days postinoculation with *E. falciformis* oocysts. ×100.

numbers of IgAc were approximately 2.5 times that of controls (Fig. 7). Significantly more IgAc were present 11 and 13 DPI in the large intestines of naive mice than in those of control mice.

In the large intestine of immune mice challenged with *E. falciformis,* an anamnestic IgA response occurred that appeared earlier and with greater magnitude than the primary response (Fig. 7). Peak numbers of IgAc were present at 9 DPC in immune mice. IgAc in the large intestine progressively increased in number from 6 hr to 9 DPC during which time they were 2–3 times more numerous than those in control mice (Fig. 7). At 9 DPC, there were significantly more IgAc in immune than in naive mice. At 11 and 13 DPC, even though the numbers of IgAc in immune mice had declined, they were still significantly greater than that of control mice (Fig. 7).

An IgAc response was observed in the MLN of both naive and immune mice, but no anamnestic IgAc response occurred in immune mice (Fig. 8). At 1–5 DPC, there were no significant differences in numbers of IgAc in the MLN of immune, naive, or control mice. At 9 and 11 DPC, immune or naive mice had significantly more (between 3.5 and 8.4 times) IgAc than did control mice, and naive mice had significantly more IgAc than immune mice. At 7 and 13 DPC, immune and control mice had similar numbers of IgAc, whereas naive mice had significantly more IgAc.

IgG and IgM lymphocyte responses

The kinetics of the primary and anamnestic IgGc responses in the large intestine (Fig. 9) were similar to those of the IgAc responses in this tissue (Fig. 7). The greatest numbers of IgGc were present in the large intestine of naive mice at 11

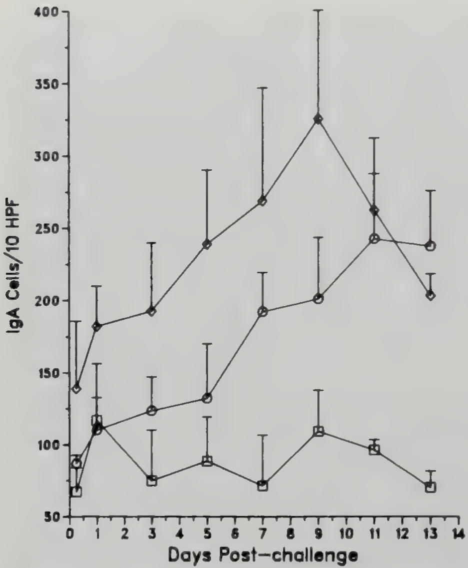


FIGURE 7. IgA lymphocyte response in the large intestine of naive (○) and immune (◇) mice at 6 hr to 13 days after challenge with oocysts of *E. falciformis*; control (noninfected) mice (□). Bars represent ±SD.

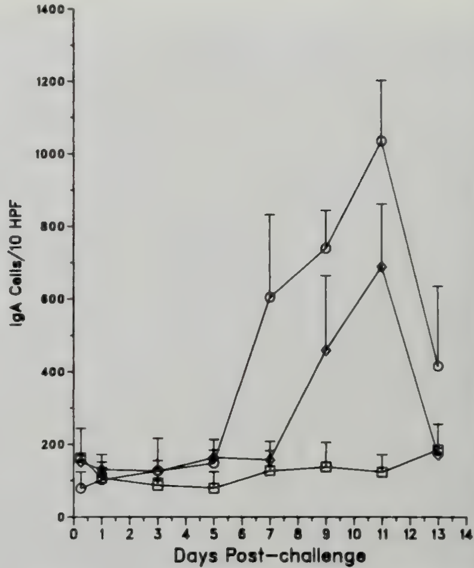


FIGURE 8. IgA lymphocyte response in the mesenteric lymph nodes of naive (○) and immune (◇) mice at 6 hr to 13 days after challenge with oocysts of *E. falciformis*; control (noninfected) mice (□). Bars represent ±SD.

and 13 DPI, being 5.4–7.1 times that of controls (Fig. 9). The numbers of IgGc peaked on 9 and 11 DPC in the large intestines of immune mice and were 10.8–30.4 times that of controls. At 3–13 DPC, the number of IgGc in immune mice was significantly greater than in controls (Fig. 9).

The MLN of immune mice did not show an anamnestic IgGc response to parasite challenge (Fig. 10). The primary IgGc response in the MLN peaked on 11 DPI at 18.7–43.2 times that of controls. Numbers of IgGc in immune mice also peaked on 11 DPC at 12.1–38 times controls (Fig. 10). No significant differences in the numbers of IgMc occurred in the large intestine or MLN of control, naive, or immune mice (data not shown).

DISCUSSION

The immune response to eimerian parasites appears to involve both antibody and cell-mediated mechanisms. Evidence that the development of immunity to coccidiosis is predominantly T cell-mediated has been based upon detection of delayed-type hypersensitivity (Rose, 1977; Klesius and Giambrone, 1984), the ability of immune lymphocytes or lymphokines to transfer resistance to infection (Liburd et al., 1972,

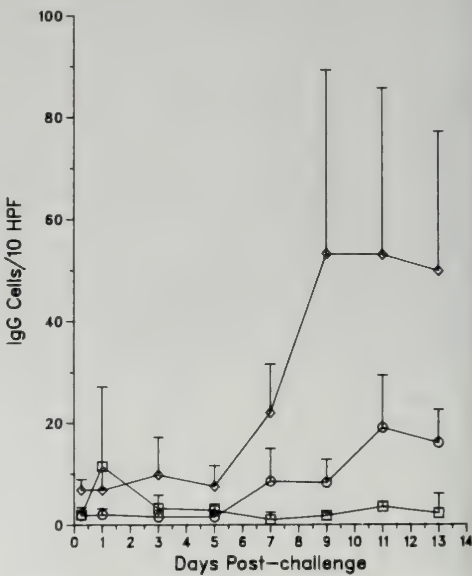


FIGURE 9. IgG lymphocyte response in the large intestine of naive (○) and immune (◇) mice at 6 hr to 13 days after challenge with oocysts of *E. falciformis*; control (noninfected) mice (□). Bars represent ±SD.

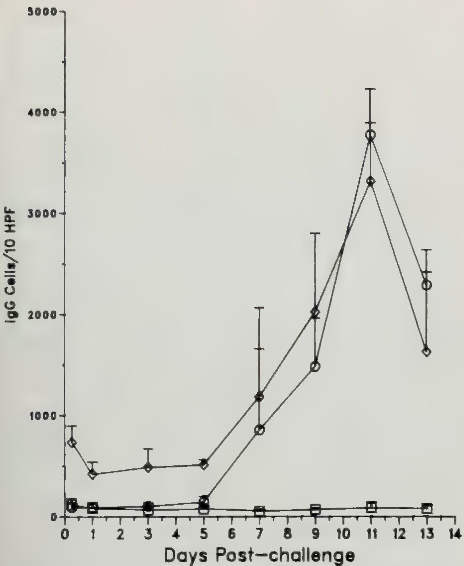


FIGURE 10. IgG lymphocyte response in the mesenteric lymph nodes of naive (○) and immune (◇) mice at 6 hr to 13 days after challenge with oocysts of *E. falciformis*; control (noninfected) mice (□). Bars represent ±SD.

1973; Klesius and Giambrone, 1984), the ability of lymphokines to inhibit parasite development (Speer et al., 1985; Hughes et al., 1987), and the failure of athymic mice and rats to acquire resistance to *Eimeria* species (Mesfin and Bellamy, 1979a, 1979b; Rose and Hesketh, 1979; Rose et al., 1979). However, because the production of IgA is dependent upon T-cell help (Elson et al., 1979), athymic rodents cannot be used to unequivocally exclude the importance of antibodies in resistance to coccidiosis.

Most attempts to passively transfer immunity to *Eimeria* spp. with serum have failed (Becker et al., 1935; Senger et al., 1959; Fitzgerald, 1964), although some resistance to *E. nieschulzi* and *E. maxima* can be conferred to naive hosts by the repeated injection of serum from infected rats (Rose and Hesketh, 1979) or chickens (Rose, 1971, 1974), respectively. All of these antibody transfer studies, however, involved the intravenous or intraperitoneal administration of a circulating antibody to protect against a parasite of the intestinal mucosa. This method of passive immunization will protect recipients against an intravenous parasite challenge (Long and Rose, 1965), but is not as effective against an oral challenge. Because most *Eimeria* spp. have no known extraintestinal stages, transfer of immune serum is apt to provide little or no protection. There is little or no serum leakage into the mucosa due to the inflammatory processes associated with coccidial infections (Rose and Long, 1969; McKenzie et al., 1986), and it is more likely that anti-parasite antibodies are stimulated and produced locally. Thus, both specific antibody and cell-mediated immune responses may be necessary to provide protective immunity against coccidiosis.

In earlier studies, we found parasite-specific IgA in the intestinal lumen and mucosa of mice immune to *E. falciformis* (Douglass and Speer, 1985; Whitmire and Speer, 1986). IgA-containing intestinal mucus from immune mice agglutinated sporozoites, whereas that from nonimmune mice did not (Douglass and Speer, 1985). In the present study, we found that IgAc were concentrated in the apical portion of the lamina propria of the large intestine in naive and immune mice challenged with *E. falciformis*. Even though the specificity of these lymphocytes is unknown, challenge with *E. falciformis* clearly induces migration of IgAc to the site of infection.

IgAc also represented the largest isotype B-cell population (about 95%) in naive or immune mice inoculated with *E. falciformis*. Because *E. falciformis* parasitizes enterocytes, localization of IgAc in the upper part of the intestinal mucosa may provide greater contact between antibodies and parasites. IgA may have access to parasites within enterocytes because IgA is transported in vesicles across these cells and secreted onto the luminal surface (Nagura et al., 1979; Brandtzaeg, 1981). Parasite-specific secretory IgA (SIgA) may function in "immune exclusion" (Tomasi and Grey, 1972; Heremans, 1974) by complexing with the surface of extracellular invasive stages such as sporozoites and merozoites. Davis and Porter (1979) suggested that SIgA might participate in immunity to coccidiosis by attaching to the surface of sporozoites and merozoites and inhibiting or delaying their penetration of enterocytes. Although similar numbers of *E. falciformis* sporozoites were found in the intestinal epithelial cells of immune and nonimmune-infected mice, the sporozoites in immune mice degenerated or did not continue development normally (Mesfin and Bellamy, 1979b). Thus, as Davis and Porter (1979) also suggested, those parasites that are successful in penetrating enterocytes may be sufficiently altered so that they are incapable of further development.

The predominance of the IgAc response to infection with *E. falciformis* may prevent immunologically induced cytopathic effects in the gut of immune animals. Both IgG and IgM bind complement, but IgA does not. Changes in enterocyte membrane proteins have been found during infection with *Eimeria necatrix* (Pasternak and Fernando, 1984), indicating that parasite antigens may occur on host cell surfaces as is the case with other apicomplexans (Schmidt-Ullrich and Wallach, 1978; Newbold et al., 1982; Perkins, 1982; Speer and Burgess, 1987). Complement-fixing antibodies to these altered proteins could lyse enterocytes, causing further damage to host tissues. Therefore, IgA antibodies may act to block the binding to mucosal cells of other potentially destructive immunoglobulins.

The number of IgGc in the normal mouse gut was only 3.6% of the total of the 3 immunoglobulin classes examined. A relatively small increase in IgGc occurred in the lamina propria during *E. falciformis* infection, and most of the cells accumulated at the base of the mucosa. Localization of IgGc away from the intestinal epithelium may reduce the amount of complement-fixing antibody in the vicinity of potentially vulnerable host cells. Also, parasite-specific IgG antibodies at the base of the mucosa may prevent extension of the parasite into the submucosa and extraintestinal tissues.

IgMc comprised the smallest portion (1.4%) of immunoglobulin-containing lymphocytes in the large intestine of control mice. Naive or immune mice exhibited no IgMc response to *E. falciformis* nor any change in the distribution of IgMc in the lamina propria of the large intestine.

Primary and anamnestic IgAc and IgGc responses to *E. falciformis* occurred in the large intestine of naive and immune mice. The heightened anamnestic B-cell response is likely due to the presence of memory cells, generated during the initial infection, that respond quickly to parasite challenge. They may reside in the mucosa after initial formation, but are more likely to recirculate because of their small size (Phillips-Quagliata et al., 1983). When stimulated by antigenic challenge, specific memory cells may extravasate, develop into plasma cells, and become lodged in the lamina propria. This may account for the rapid increase in IgAc numbers in immune mice in this study as well as for the anamnestic response in intestinal IgA titers seen in mice with acquired resistance to *E. falciformis* (Douglass, 1983).

Although there was a significant increase in the number of IgGc in the large intestine of infected mice, cells of this isotype represented only a small fraction of the B-cell response. IgGc exhibited primary and anamnestic responses after parasite challenge indicating that parasite-specific memory cells of the IgG class may also be generated during the primary infection.

Numbers of IgAc and IgGc in the MLN peaked at 11 DPC in both naive and immune mice. None of the B-cell isotypes exhibited an anamnestic response in the MLN, and immune mice actually had reduced B-cell responses in the MLN after parasite challenge when compared with naive mice. Because most memory cells migrate to the gut after generation in the MLN (McWilliams et al., 1975, 1977), it is likely that an anamnestic B-cell response would be difficult to detect in the nodes of mice after parasite challenge. The depression in numbers of B cells in the MLN of challenged, immune mice is difficult to explain, but may be the result of a decrease in the amount of available antigen providing stimulation in the gut. If local immune responses in previously infected animals cause rapid reduction in numbers of parasites after the challenge inoculation, then fewer cells would be stimulated in the lymphoid nodules, and fewer blasts would, therefore, migrate to the MLN.

Almost 5 times more IgGc than IgAc were present in MLN of immune mice, but most of these evidently did not migrate to the large intestine because relatively few IgGc were observed there. Mature IgGc might remain in the MLN and secrete antibody into the circulation, rather than migrating to the intestinal mucosa.

This study has shown that there are significant local accumulations of IgAc and IgGc in the large intestine and MLN in response to *E. falciformis*. The presence of large numbers of IgAc in the lamina propria of naive and immune mice indicates that IgA may have a functional role in acquired resistance to *E. falciformis*. However, relative numbers of parasite-specific IgAc and IgGc must be determined before their participation in protective immunity to *E. falciformis* can be accurately assessed.

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DISCULICEPS GALAPAGOENSIS N. SP. (LECANICEPHALIDEA: DISCULICEPITIDAE) FROM THE SHARK, *CARCHARHINUS LONGIMANUS*, WITH COMMENTS ON *D. PILEATUS*

Anne Marie Nock and J. N. Caira

Ecology and Evolutionary Biology, University of Connecticut, U-43, Rm. 312, Storrs, Connecticut 06268

ABSTRACT: *Disculiceps galapagoensis* n. sp. from the spiral valve of an oceanic whitetip shark, *Carcharhinus longimanus*, collected near the Galapagos rift is described and figured. It differs from *D. pileatus* (Linton, 1891) Joyeux and Baer, 1935, the only other species currently recognized in the genus, as follows: The collar is much wider in proportion to the cushion, the cushion tapers gradually to meet the collar rather than meeting it abruptly, and the vas deferens is much less extensive and convoluted. In addition, scanning electron microscopy reveals that the surface of the cushion in the new species bears a regular pattern of pores arranged in numerous parallel rows, whereas the cushion of *D. pileatus* apparently does not. The family diagnosis of the Disculicepitidae, and the description of *D. pileatus* are emended. The systematic position of the genus is discussed and it is recommended that, for the present, the group remains as a family in the order Lecanicephalidea.

Pintner (1928) erected the family Discocephalidae for the single distinctive species, *Discocephalum pileatus* Linton, 1891, collected from *Carcharhinus obscurus* (Le Sueur) at Wood's Hole, Massachusetts. Joyeux and Baer (1936) recognized that the name Discocephalidae Pintner, 1928, was a homonym and suggested the replacement names *Disculiceps pileatus* (Linton, 1891) Joyeux and Baer, 1935, and Disculicipitidae Joyeux and Baer, 1935, for the species and family names, respectively. Wardle and McLeod (1952) noted that the family name should be Disculicepitidae. Butler (1984) recognized that, because this is a case of family-group name homonymy, the names suggested by Joyeux and Baer (1935) are not valid until the issue has been ruled upon by the International Commission for Zoological Nomenclature. In order to validate these names, a petition to this effect has been submitted for consideration by the Commission (Caira, 1987). Article 80(a) of the International Code of Zoological Nomenclature states that when a case is under consideration by the Commission, existing usage is to be maintained until the Commission publishes its ruling on the issue. Because the names Disculicepitidae and *Disculiceps pileatus* are in current usage (Schmidt, 1986), these names are used throughout this paper.

Examination of *Carcharhinus longimanus* (Poey) caught near the Galapagos rift revealed a new species of *Disculiceps*, which is described below. Because of some uncertainty associated with details of the morphology of *D. pileatus*, it was compared with the new species. The description of *D. pileatus* and the diagnosis of the Disculicepitidae are emended. Both species were examined with scanning electron microscopy (SEM) for the first time.

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MATERIALS AND METHODS

Specimens of the new species were collected from an individual of *Carcharhinus longimanus* caught by hook and line from ship, near the Galapagos rift, in November 1979. The entire spiral valve with specimens attached was removed and fixed.

The whereabouts of the type material of *Disculiceps pileatus* is at present uncertain. Linton (1891) gave no indication of the disposition of the type specimens. Inquiry at the U.S. National Museum Helminthological Collection revealed that 3 lots (Nos. 7694, 35052, 34986) of *D. pileatus*, collected at Wood's Hole, Massachusetts, were deposited there. No. 7694 consists of a single mounted specimen from *Carcharhinus milberti* Valenciennes (=*C. plumbeus* (Nardo)), determined by Linton (therefore a metatype). No. 35052, which lacks a scolex, from *C. milberti* (=*C. plumbeus*), and No. 34986 from *C. obscurus*, consist of unmounted material collected and determined by G. A. MacCallum. In addition, 2 specimens of *D. pileatus* collected from *C. plumbeus* off Rhode Island were examined in the present study.

Some specimens prepared as whole mounts were hydrated in a series of alcohols, stained in Gill's hematoxylin, and dehydrated in a series of alcohols; others were stained in Semichon's acetocarmine and dehydrated in a series of alcohols. All specimens were cleared in xylene and mounted in Canada balsam. For cross or longitudinal histological sections specimens were embedded in paraplast, sectioned at 10-μm intervals with an American Optics rotary microtome, stained in Gill's hematoxylin and eosin, cleared in xylene, and mounted in Canada balsam. Three scolices of the new species and 1 of *D. pileatus* were prepared for SEM as

follows: They were dehydrated in a series of alcohols and critical point dried in wire baskets using liquid CO_2. The dried worms were mounted on stubs with Apiezon W100 wax, sputter coated with gold, and examined with a Coates and Welter Field Emission Scanning Electron Microscope.

Illustrations were prepared with the aid of a drawing tube. Measurements are in μm unless otherwise stated. Ranges are given in the text followed in parentheses by the mean, the standard deviation, the number of worms examined, and the total number of observations when more than 1 structure per worm was examined.

Representative specimens of *D. pileatus* were deposited at the USNM Helminthological Collection (No. 79716) in Beltsville, Maryland and at the H. W. Manter Laboratory (Nos. 19994 and 19995) in Lincoln, Nebraska.

RESULTS

Disculicepitidae Joyeux and Baer, 1935, emend.

The following diagnosis is based on those of Yamaguti (1959) and Schmidt (1986) but is emended to include information obtained in the present study:

Family diagnosis: Scolex without suckers or hooks, clearly divided into anterior cushion and posterior collar with corrugated surface. Neck present. Strobila flat, anapolytic, increasing in breadth towards mature segments, then narrowing towards gravid segments. Segments acraspedote, short, crowded anteriorly. Testes racemose, occupying most of preovarian area. Cirrus pouch well developed; cirrus with minute spines, opening behind vagina in common, submarginal, genital pore. Ovary bilobed, at posterior end of segment. Seminal receptacle absent. Uterus median, with lateral branches, opening in gravid segments along median line by longitudinal dehiscence. Vitelline follicles circumcortical. Eggs in packets of 6–8 onchospheres. Parasites of elasmobranchs.

Generic diagnosis: Disculicepitidae, with characters of the family.

Disculiceps pileatus (Linton, 1891), emend.
(Figs. 4–7)

The description of *D. pileatus* should be corrected to contain the following information: Segments acraspedote; cirrus bearing minute spines; cirrus sac and vagina opening into common, submarginal, genital aperture; vitellaria circumcortical.

Disculiceps galapagoensis n. sp.
(Figs. 1–3, 8–10)

Description (based on 3 entire worms, 6 partial worms with scolices, and several partial strobila): Worms 70–96 mm (82.8 ± 12.8; 3) long; 376–393 (384 ± 86; 3) segments per worm; acraspedote; anapolytic; genital pore submarginal, in anterior half of segment, irregularly alternating. Scolex bipartite, consisting of anterior cushion and posterior collar; cushion globose, smooth in gross appearance, 345–1,500 (1,051 ± 353.2; 9) long by 525–3,270 (1,797.2 ± 916.9) wide; collar with corrugated surface, 345–1,500 (853.3 ± 430.9; 9) long by 480–2,070 (1,340 ± 497.2; 8) wide; ratio of cushion length to collar length 0.86–1.93:1 (1.07:1 ± 0.47; 8); ratio of cushion width to collar width 1.09–2.03:1 (1.5:1; 0.31; 7); suckers entirely lacking from both cushion and collar. Neck 325–1,650 (1,191 ± 389; 5) long.

Strobila narrows toward gravid segments. Immature segments wider than long. Mature segments 420–1,395 (837 ± 220; 8; 49) long by 1,635–5,145 (3,527.9 ± 2,281; 8; 49) wide; ratio of width to length 1.9–5.6:1 (3.9:1 ± 0.88; 8; 49). Gravid segments 1,200–3,360 (1,886 ± 540; 6; 38) long by 2,250–4,455 (3,317 ± 636.3; 6; 39) wide; ratio of width to length 1.03–3.4:1 (1.94:1 ± 0.78; 6; 39). Testes numerous, larger in gravid segments than in mature segments, distributed in semblance of parallel, lateral rows, and throughout segment anterior to ovary, interrupted by cirrus pouch. Vas deferens coiled; cirrus pouch 300–885 (478.4 ± 196.5; 10; 29) long by 60–300 (174 ± 81; 10; 29) wide; cirrus 450–705 (591 ± 77; 4; 7) long by 150–210 (176 ± 27; 4; 4) wide, armed with minute spines. Ovary posterior, bilobed, 120–285 (175.7 ± 25.5; 5; 9) long by 3,060–4,530 (3,763 ± 627.2; 5; 9) wide. Mehlis' gland postovarian; vagina extending from submarginal genital pore, along anterior margin of cirrus pouch to median line of segment, then posterior to ovarian bridge. Uterus median, branched, and opening medially with dehiscence in gravid segments. Remaining organs of female and male reproductive systems atrophied in gravid segments. Preformed uterine pores lacking. Vitelline follicles circumcortical 12.2–24 (19.5 ± 10.3; 6; 40) wide by 6.1–18.3 (17.9 ± 10.4; 6; 40) long, extending laterally beyond testes, interrupted by cirrus pouch. Longitudinal excretory ducts inconspicuous. Longitudinal muscles present as weakly developed muscle fibers. Eggs in packets of 6–8 onchospheres.

Host: *Carcharhinus longimanus* (Poey), oceanic whitetip shark.

Locality: Galapagos rift, 00°48′N, 86°13′W.

Site of infection: Spiral valve.

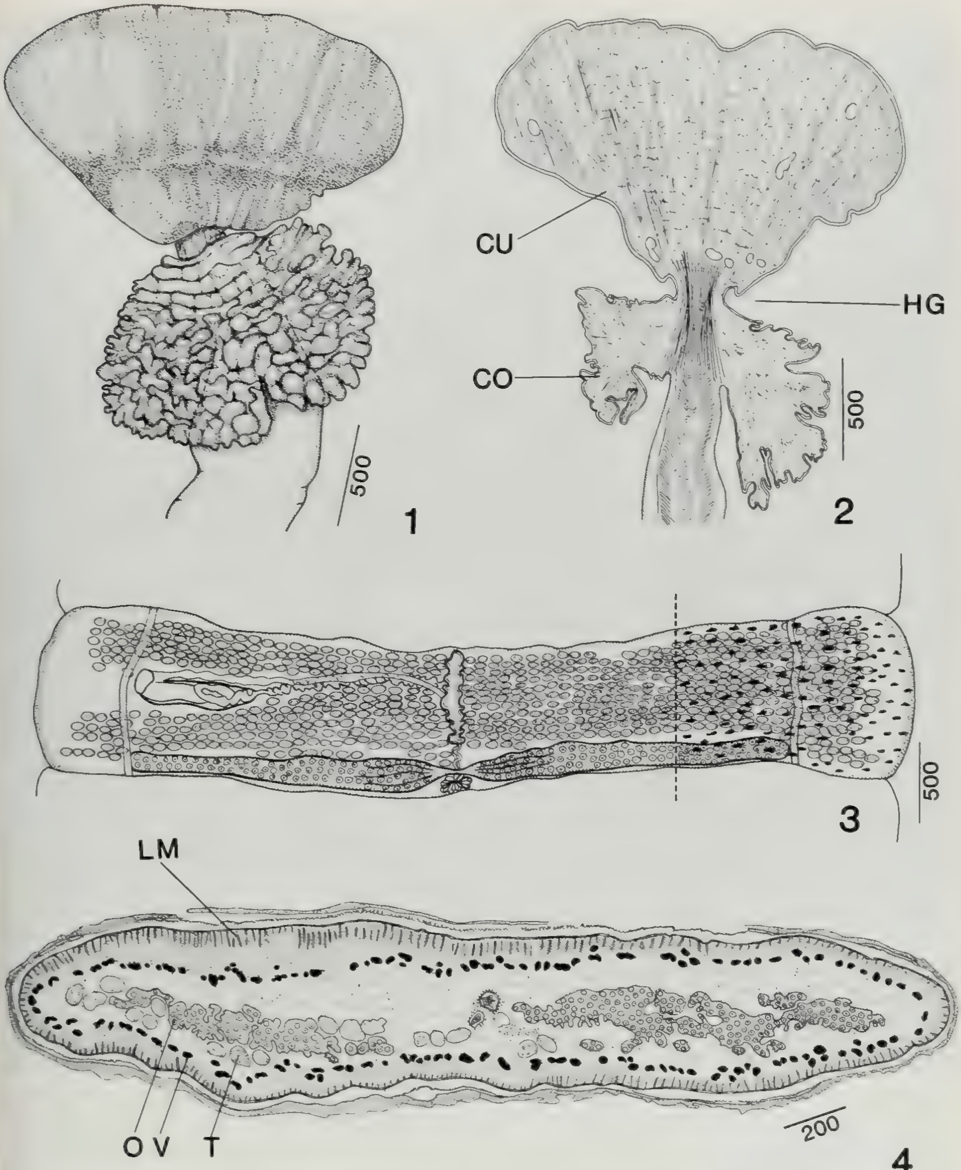
Holotype: USNM Helm. Coll. No. 79714.

Paratypes: USNM Helm. Coll. No. 79715; HWML Nos. 19992 and 19993.

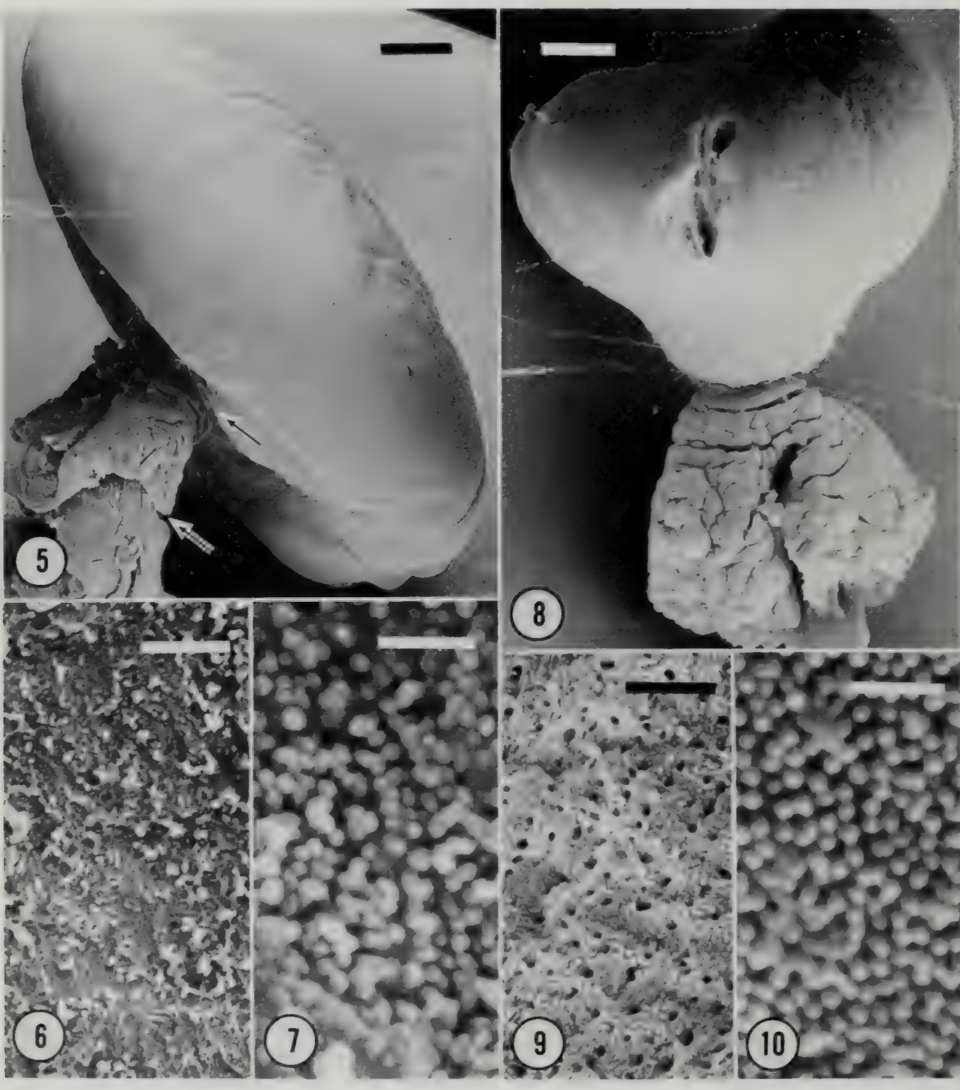
Etymology: This species is named after the type geographic locality.

Comparisons

Members of the family Disculicepitidae differ from the members of all other families of Lecanicephalidea in that both the anterior and posterior portions of their scolices lack suckers. At present, the family Disculicepitidae contains the single genus *Disculiceps*. *Disculiceps galapagoensis* may be distinguished from the only other species in the genus, *D. pileatus*, on the basis of the following characters: The collar is much wider in proportion to the cushion (the cushion-to-collar width ratio is 1.09–2.03:1 in *D. galapagoensis* and 3.65–4.08:1 in the specimens of *D. pileatus* examined in the present study); in *D. galapagoensis* the cushion tapers gradually towards the collar whereas in *D. pileatus* the undersurface of the cushion is very flat, and joins the collar abruptly; and the vas deferens of *D.*



FIGURES 1–4. **1–3.** *Disculiceps galapagoensis* n. sp. **1.** Whole mount of scolex. **2.** Longitudinal section through scolex. **3.** Whole mount of mature segment. Vitellaria are circumcortical throughout the segment, but are illustrated only to the viewer's right of the dashed line. **4.** *Disculiceps pileatus.* Cross section through ovary of mature segment. CO = collar, CU = cushion, HG = horizontal groove, LM = longitudinal muscle, O = ovary, T = testis, V = vitellaria.



FIGURES 5–10. **5–7.** Scanning electron micrographs of scolex of *Disculiceps pileatus*. **5.** Entire scolex. Collar extends between arrows. Scale bar = 200 μm. **6.** Magnified view of cushion. Scale bar = 5 μm. **7.** Magnified view of collar. Scale bar = 0.5 μm. **8–10.** Scanning electron micrographs of scolex of *Disculiceps galapagoensis* n. sp. **8.** Entire scolex (cushion is damaged slightly). Scale bar = 200 μm. **9.** Magnified view of cushion. Scale bar = 5 μm. **10.** Magnified view of collar. Scale bar = 0.5 μm.

pileatus is much more extensive and convoluted than that of *D. galapagoensis.*

Scanning electron microscopy of the scolices reveals that the surface of the collar is similar in both species; numerous, densely packed, tiny protrusions (resembling microvilli) are present throughout the surface of the collar (Figs. 7, 10). However, differences in the surface of the cushion were observed between the 2 species. The cushion of *D. galapagoensis* possesses a regular pattern of pores arranged in numerous, parallel rows (Fig. 9), whereas there is no evidence of regular pores on the surface of the cushion of *D. pileatus* (Fig. 6).

The scolex of *D. galapagoensis* was attached to the internal surface of the spiral valve in a manner similar to that described by Pintner (1928) for *D. pileatus*. The cushion of the scolex was entirely buried into the host tissue, but the posterior portion of the collar was clearly visible extending from the mucosa. There was a definite circular host encapsulation response around the cushion of the scolex such that removal of the worms from the host tissue was very difficult.

DISCUSSION

Linton (1891) described 2 varieties among the type material of *D. pileatus*. Variety α, of which there were 2 specimens, had a disc or cushion that was entire, whereas variety β, of which there were also 2 specimens, had a disc or cushion with a profound lateral notch. Of the 4 scolices of *D. pileatus* we examined, 1 (USNM No. 34986) possessed what we assumed to be a lateral notch. When examined apically, the smooth, circular border of the cushion was interrupted by a conspicuous lateral indentation. This specimen also seemed to possess a much shallower secondary indentation directly opposite the first. Of the 9 scolices of *D. galapagoensis* we examined, none was seen to possess any evidence of a lateral notch. Owing to the lack of material of both species, it is difficult to determine the significance of the lateral notch. However, we suspect that because the cushion of *D. pileatus* is much larger in proportion to the collar than in *D. galapagoensis*, the cushion in the former tends to bend more readily, and perhaps the notch is the result of stress on the musculature of the cushion.

In one of his specimens of variety α Linton (1891) noticed that, for a short distance immediately posterior to the collar, the body was double. We found this same phenomenon in one of the specimens of *D. galapagoensis* (HWML No. 19992). The strobila appears to be split in a longitudinal line down its center for a distance of approximately 3.5 mm. In both cases the phenomenon occurred in immature segments. However, it would be interesting to investigate the effects of such a split on reproductive processes in these segments as they mature.

The material of *Disculiceps* available to Pintner (1928) consisted of specimens from the *Valdivia* explorations, collected from *Carcharias lamia* (=*Carcharodon carcharias* (L.)). Pintner did not list actual geographic localities but indicated that the specimens were collected from Stations 228 and 268. In the report published on the German deep sea expedition *Valdivia* (Schott, 1902) the following coordinates were listed for these stations: Station 228—2°39′N, 65°59′E; and Station 268—9°6′S, 53°41′E. Thus, both stations were in the Indian Ocean; Station 228 was west of the Maldive Islands, and Station 268 was near the Seychelles. Pintner (1928, p. 60) was uncertain as to whether or not his material represented the same species as that described by Linton (1891) from the Woods Hole region. In fact, he suggested that it might be better to consider his material as a different species or even genus than the material of Linton (1891), however, he did not officially designate it so. The scolex/collar proportions of the specimen illustrated in Pintner's figure 8 resemble *D. galapagoensis* much more closely than *D. pileatus*. We have been unable to locate Pintner's specimens of *Disculiceps*. Until his specimens can be located or until additional collections can be made from the same geographic localities, the identity of Pintner's material of *Disculiceps* will remain uncertain.

Our results allow us to comment on several morphological details about which there is some disagreement. The segments of *D. pileatus* have been described as craspedote (Perrenoud, 1931; Wardle and McLeod, 1952) and acraspedote (Pintner, 1928; Schmidt, 1986). In both species of *Disculiceps* examined in the present study, the segments were acraspedote. The vitellaria of *D. pileatus* have been described as consisting of a compact mass located posterior to the ovary (Linton, 1891; Southwell, 1925; Wardle and McLeod, 1952; Wardle et al., 1974), as well as being essentially circumcortical in distribution (Pintner, 1928; Perrenoud, 1931; Euzet, 1959). In the present study several segments of both species of *Disculiceps* were sectioned and the vitellaria in these segments were circumcortical. The genital apertures of *D. pileatus* were originally reported to be marginal by Linton (1891) but were described as submarginal by Pintner (1928), Perrenoud (1931), and Schmidt (1986). We found the genital apertures of both species of *Disculiceps* to be submarginal. With the exception of Pintner (1928), the cirrus of *D. pileatus* has consistently been described as lacking spines, but we found minute spines in sections of the cirrus in both species.

The discovery of a second species in the previously monotypic *Disculiceps* aids in establishing the identity of the genus, but does not necessarily assist in the clarification of the systematic position of the taxon. Joyeux and Baer (1935)

recognized this group as a family in the order Tetraphyllidea. Wardle and McLeod (1952) considered it to be sufficiently distinct from the Tetraphyllidea to justify the establishment of a special order, the Disculicepitidea. However, the majority of authors (Pintner, 1928; Perrenoud, 1931; Yamaguti, 1959; Schmidt, 1986) placed the group as a family in the order Lecanicephalidea.

Because scolex bothridia are entirely lacking, these species should not be placed in the order Tetraphyllidea. The group should probably not be considered as an order in its own right, because its characteristics satisfy the current diagnosis of the order Lecanicephalidea (see Schmidt, 1986): The scolex is divided transversely into an anterior and a posterior region by a horizontal groove. Pintner (1928) presented a large amount of evidence however, to suggest that *Disculiceps* should be separated (at least at the family level) from such lecanicephalans as *Tylocephalum* Linton, 1890 and *Lecanicephalum* Linton, 1890, because the 2 regions of the scolex are not homologous among these groups and thus the possession of a bipartite scolex is of little significance.

Our current recommendation is to retain the group as a family in the Lecanicephalidea until further comparative studies that would facilitate a cladistic analysis of the members of that order can be performed. In addition, it would be particularly interesting to examine the 2 regions of the scolex of other members of the Lecanicephalidea with scanning electron microscopy in light of the details discovered in the present study.

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We express our appreciation to Dr. F. Whittaker of the University of Louisville for the specimens of *D. galapagoensis* that were collected by A. R. Velasco. In addition, we thank Ms. J. Gibb, and others at Mystic Marine Life Aquarium in Connecticut, for providing us with specimens of *D. pileatus*. We also thank Dr. J. R. Lichtenfels of the U.S. National Parasitology Institute for lending specimens, and Dr. A. Wachtel and Mr. J. Romanov of the University of Connecticut for their assistance with the scanning electron microscopy. Lastly, we are truly indebted to Dr. M. Lefor for translating much of Pintner (1928) from the original German.

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MYOBIIDAE (ACARINA, TROMBIDIFORMES) ASSOCIATED WITH MINOR FAMILIES OF CHIROPTERA (MAMMALIA) AND A DISCUSSION OF PHYLOGENY OF CHIROPTERAN MYOBIID GENERA

Kimito Uchikawa
Department of Parasitology, Shinshu University School of Medicine, Matsumoto 390, Japan

ABSTRACT: Three new genera, *Natalimyobia* n. gen., *Furipterobia* n. gen., and *Mizopodobia* n. gen., are erected for the myobiid mites associated with the chiropteran families Natalidae, Furipteridae, and Myzopodidae, respectively. The definitions for the genera *Nycterimyobia, Thyromyobia,* and *Mystacobia,* previously known only from one sex, are refined with the description of the opposite sex. Two different types of myobiid genera, one being represented by larger elongate forms and the other by smaller rounded forms, often share hosts within a chiropteran family. A larger elongate myobiid genus is associated exclusively with each chiropteran family. Phylogenetic relationships among 16 larger-sized myobiid genera, including the 3 new ones, are deduced based primarily on the structure of female genitalia and secondarily on the formation of legs and chaetotaxy of idiosoma and legs. These relationships are regarded as being parallel to those of the chiropteran host families.

Permanent or holostadial ectoparasites of the family Myobiidae are thought to be excellent indicators of the taxonomy and phylogeny of their chiropteran hosts (Uchikawa and Harada, 1981), which lack the well known indicatory parasites, Mallophaga and Anoplura (Mayr, 1957). Some current problems in host sytematics have recently been elucidated on the basis of knowledge of the myobiids specific to bats of the families Miniopteridae (Uchikawa, 1985a, 1985b) and Pteropodidae (Uchikawa, 1986a, 1986b).

The purpose of this study was to examine representatives of several families of bats for which no myobiids or myobiids of only one sex were known. A second purpose was to incorporate this new information into a discussion of the phylogeny of the chiropteran myobiids, which is thought to parallel that of the host.

MATERIALS AND METHODS

Alcoholic and skin specimens of bats of the families Rhinopomatidae, Craseonycteridae, Megadermatidae, Noctilionidae, Natalidae, and Myzopodidae, minor families which had not previously yielded any myobiid mites, and Nycteridae, Furipteridae, Thyropteridae, and Mystacinidae, families in which myobiids of only one sex were known, were examined in the following museums: British Museum (Natural History), London (BMNH), Museum National d'Histoire Naturelle, Paris (MNHN), Rijksmuseum van Natuurlijke Histoire, Leiden (RMNH), Forschungs-Institut Senckenberg, Frankfurt (SMF), the American Museum of Natural History, New York (AMNH), and the U.S. National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM), using methods outlined in previous papers (Uchikawa, 1986a, 1986b).

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Myobiids obtained from Rhinopomatidae and Noctilionidae are not discussed as it is uncertain whether they are true parasites of those hosts. Although a number of bats of specimens in the families Craseonycteridae (1 species, 30 individuals) and Megadermatidae (4 genera and 6 species, 43 individuals) were examined, no myobiid mites were found. The present paper thus includes new taxa of myobiid mites from the chiropteran families Natalidae, Furipteridae, and Myzopodidae and the previously unknown sex of myobiids found on Nycteridae, Thyropteridae, and Mystacinidae. A discussion of the phylogeny of the chiropteran Myobiidae, including new taxa, follows. Measurements presented in the text are in μm.

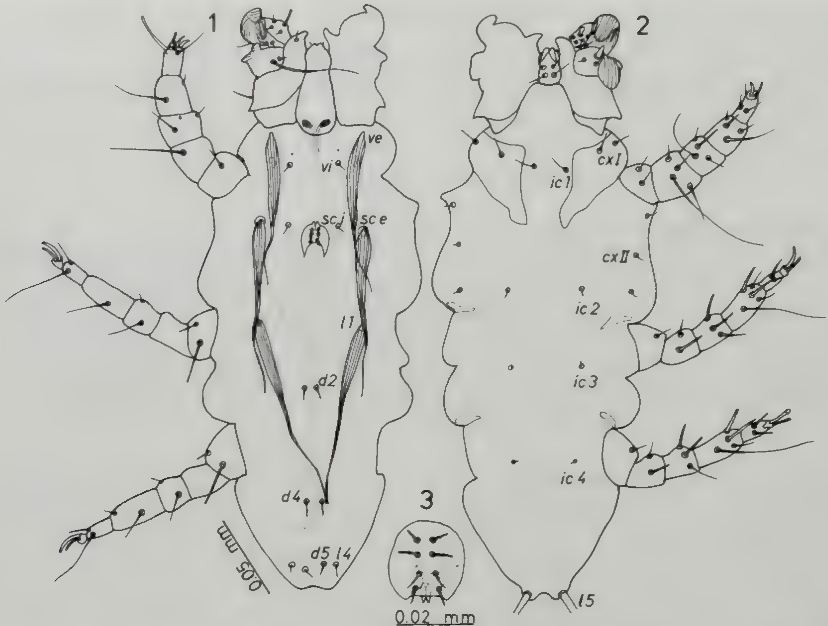
DESCRIPTION

The family Nycteridae, or hollow-faced bats, consisting of 1 genus and about 15 species is known to harbor a myobiid of the genus *Nycterimyobia* that has been defined on the basis of the female and nymph (Fain, 1972a). In the present study, both sexes of a new species and females of the known species were found. Definition of the genus *Nycterimyobia* is supplemented with discovery of the male and a new species is described.

Nycterimyobia Fain

Nycterimyobia Fain, 1972a: 61; 1978a: 113.

Gnathosoma elongate dorsally. Idiosoma elongate; *vi* fine and setiform; hysterosomal setae consisting of d_2, d_4, d_5, l_1, l_4, and l_5 in male and of d_{1-3}, d_5, and l_{1-5} (complete) in female; ventral setae short. Genitalia: Male genital shield rounded, bearing 4 pairs of genital setae; in female, vulvar lobes not developed, 7 pairs of genital setae (g_{1-7}, complete) present. Legs: Leg I 4-segmented, lacking striated structure ventrally on tibio-tarsus; a finger-like seta ventrally on tibio-tarsus I; trochanter I larger than other segments; claw formula 0-2-2-2, paired claws on each leg being subequal in size



FIGURES 1–3. *Nycterimyobia camerounensis* n. sp., male. **1.** Dorsum. **2.** Venter. **3.** Genital shield.

to each other; setal formula for coxal regions I–IV 2-3-0-0. Solenidia on legs I and II as in Figures 1 and 2; a long and conspicuous solenidion and, ventrally, a weakly clavate one on tibio-tarsus I; solenidion ventrally on genu I not visible.

Type species: Nycterimyobia nycteris Fain, 1972a: 61.

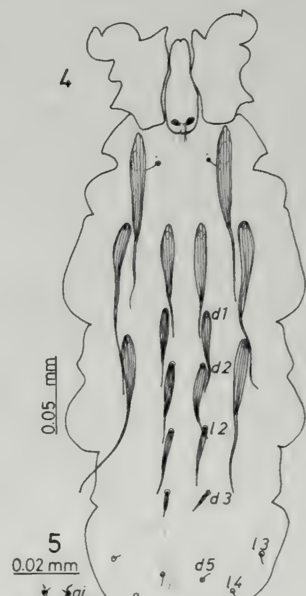
Type host: Nycteris hispida (from Angola, Fain, 1972a).

Nycterimyobia camerounensis n. sp.
(Figs. 1–5)

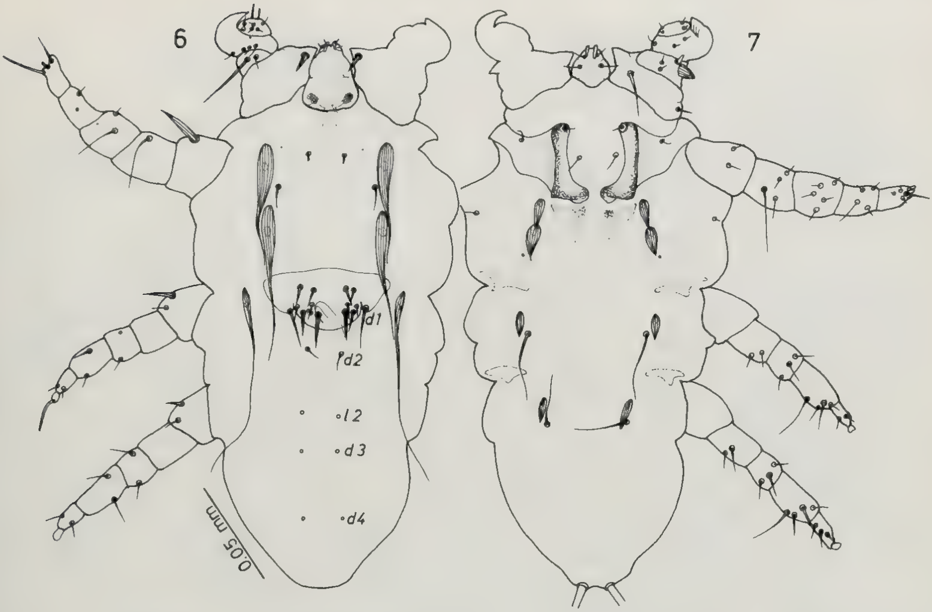
Male (Figs. 1–3): Body (gnathosoma + idiosoma) 320 (holotype)–315 (paratype) long by 135–135 wide. Dorsal setae *ve, sc e,* and l_1 expanded, striated, long and with blunt tips. Genital shield situated on slightly posterior level to bases of *sc i,* circular as in Figure 3 (outline in Fig. 1 probably deformed); penis thin and long. Nine pairs of ventral setae as in Figure 2; setae posterior to coxal regions II shorter than 3 pairs of intercoxal and coxal setae I. Chaetotaxy on legs II–IV: Trochanters 3-3-3; femora 5-2-2; genua 6-5-5; tibia 6-6-6; tarsi 5-4-4; no leg seta spatulate.

Female (Figs. 4, 5): Body 392 (allotype)–390 (paratype) long by 160–160 wide. Setae dorsally on idiosoma as in Figure 4; d_3 and l_2 short, 20–18 and 42–39 long, respectively; d_5 and l_{3-4} minute. Ventral setae of *ic* and *cx* series as in male. Gnathosoma and legs as in male.

Material examined: Holotype male, allotype female, and a male and female paratype *ex Nycteris arge,* 16 km west of Eseka, Cameroun, 24 February 1974 (host accession no. AM 236295). The holotype and allotype are deposited in the collection of the Department of



FIGURES 4, 5. *Nycterimyobia camerounensis* n. sp., female. **4.** Dorsum. **5.** Genito-anal region.



FIGURES 6, 7. *Natalimyobia handleyi* n. gen., n. sp., male. **6.** Dorsum. **7.** Venter.

Entomology, American Museum of Natural History, and the other paratypes are in the collection of the author.

Remarks: The new species is easily separable from *N. nycteris,* the generic type, by the difference in lengths of setae d_3 and l_2, which measure 20–18 and 42–39 long, respectively, in the female of *N. camerounensis* n. sp., while the corresponding setae are more than 70 and 50 long, respectively, in *N. nycteris*; d_5 and l_{3-4} are also shorter in *N. camerounensis* than in *N. nycteris.* Setal numbers on genua II–IV and tarsi II–IV are 6-5-5 and 5-4-4, respectively, in the new species, and they are 6-6-6 and 6-5-5, respectively, in *N. nycteris.*

All ventral setae are weak and both coxal setae III and IV are lacking in the genus *Nycterimyobia,* suggesting a remarkable regression of those setae from a prototypic ventral chaetotaxy. Chaetotaxy of the idiosomal venter is strictly characteristic of each genus, yet phylogenetic or evolutionary significance of the intergeneric difference in the chaetotaxy is not clear.

Fain (1978a) recorded *Nycteris hispida,* the type host, from Rwanda and Zaire, *N. arge* from Zaire, *N. damarensis* from South Africa, and an unidentified bat from Angola as the hosts of *N. nycteris.* In the present study, *N. nycteris* was taken as follows: 1 female *ex N. hispida hispida,* Nimule, Equitorial Province, Sudan, 16 March 1950 (AM 184481–4, 184486, 184487); 3 females *ex N. arge,* Chana, Zaire, 10 October 1970 (AM 233875–7). These host records suggest problems in the taxonomy of both mites and hosts, since mites of this genus are presumed to be highly host species specific. The mite from the South African *N. damarensis* and the bat from Zaire, which is frequently identified as *N. arge,* the type host of *N. camerounensis,* should be reexamined.

The family Natalidae, or funnel-eared bats, consists of 1 genus and 4 species distributed in Central and South America. This is the first report of myobiids specific to this family.

Natalimyobia n. gen.

Gnathosoma broad. Idiosoma elongate; *vi* minute in male, expanded and striated in female; hysterosomal setae consisting of d_{1-4} and $l_{1-2,5}$ in male and of d_{1-5} (complete) and $l_{1-3,5}$ in female; ic_1 flanked by hooked sclerites; 2 *cx*II, *cx*III, and *cx*IV modified to droplike setae. Genitalia: Genital shield large, semicircular, weakly sclerotized, bearing more than 7 pairs of setae in male; vulvar lobes developed but separate distally and genital setae complete (g_{1-7}) in female. Legs: Leg I 4-segmented, lacking striated structure ventrally on tibio-tarsus; all setae on tibio-tarsus I simple; trochanter I relatively larger than other segments; striated structure ventrally on femur I small and conical; anterolateral seta on all trochanters spiniform and striated; claw formula 0-2-1-1, second claw on leg II vestigial; setal formula for coxal regions I–IV 2-3-1-1. Solenidia on legs I and II as in Figure 6; 2 solenidia on tibio-tarsus I rather long.

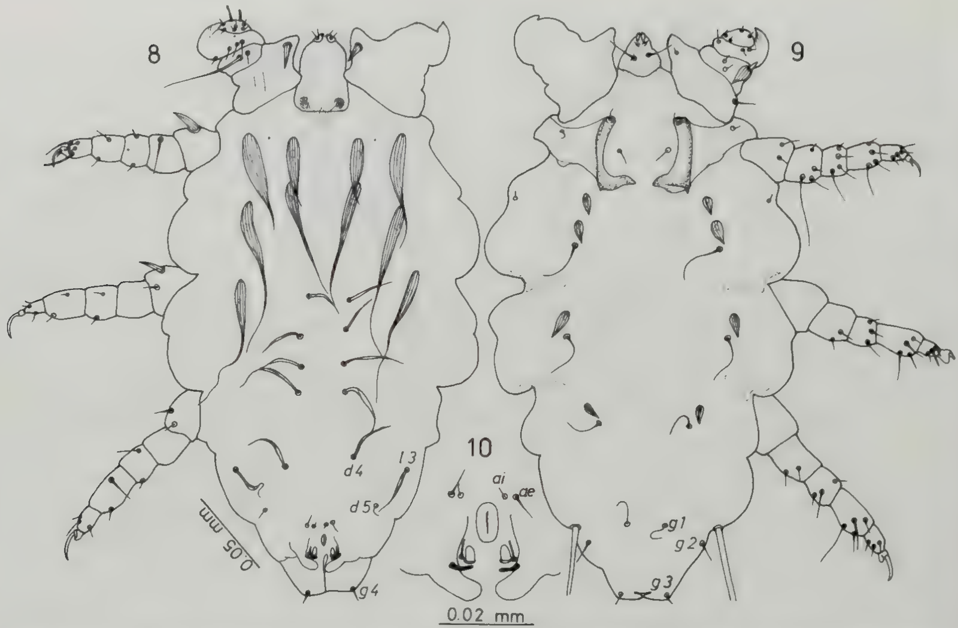
Type species: Natalimyobia handleyi n. sp.

Type host: Natalus stramineus.

Natalimyobia handleyi n. sp.

(Figs. 6–10)

Male (Figs. 6, 7): Body 250 long by 125 wide (holotype). Idiosoma widest at a level just posterior to legs II; *sc i* fine, situated mesad of bases of *sc e, ve, sc e,* and l_1 of same structure, losing thickness and gaining



FIGURES 8–10. *Natalimyobia handleyi* n. gen., n. sp., female. **8.** Dorsum. **9.** Venter. **10.** Genito-anal region.

length in this order; d_1 on genital shield. Genital shield 25 long by 57 wide, bearing 6 pairs of genital setae and, unilaterally, 2 pairs of minute setae (but bilateral asymmetry not confirmed); penis thick and ca. 70 long. Legs and leg chaetotaxy as in Figures 6 and 7; anteroventral seta on trochanter I long; some setae and claws missing but probably as in female below.

Female (Figs. 8–10): Body 313 (allotype)–320 (paratype) long by 175–180 wide. Idiosoma elliptical, widest between legs III and IV; 4 pairs of propodosomal setae and l_1 thickened and striated; d_{1-4} and l_{2-3} weakly inflated but rather slender; d_5 minute. Anal setae *ai* and *ae* almost on a line. Legs: Anteroventral seta on trochanter I minute. Chaetotaxy on legs II–IV: Trochanter 3-2-2; femora 5-2-2; genua 6-5-5; tibiae 6-6-6; tarsi 6-6-6.

Material examined: Holotype male, allotype female, and a paratype female *ex Natalus stramineus,* Canal Zone, Panama, 15 March 1961 (USNM 319071–5). The holotype and allotype are deposited in the U.S. National Museum of Natural History collection; the paratype female is in the collection of the author.

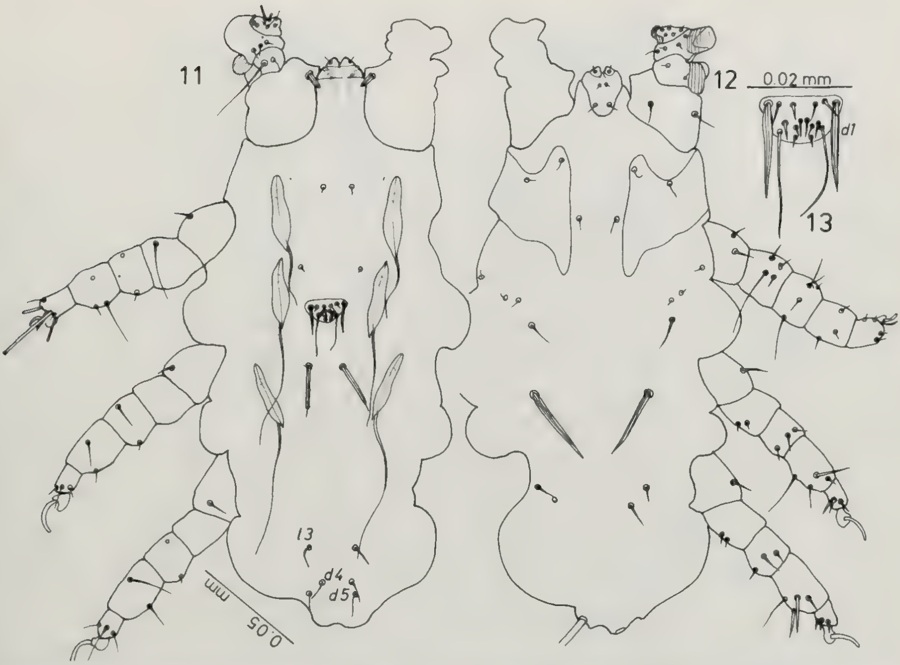
Remarks: Natalimyobia n. gen. is distinct in having leg I 4-segmented, tibio-tarsus I lacking striated structure, and droplike *cx*II and *cx*III. The nature of the submedian hysterosomal setae (d_{1-3} and l_2, Fig. 8) of the female is also unique among chiropteran Myobiidae. The vulvar lobes are large but not overlapping, suggesting that the mite is a rather primitive form.

The mite is named after Dr. Charles Handley of the Department of Mammalogy, U.S. National Museum of Natural History, Smithsonian Institution, Washington, D.C.

The family Furipteridae, or smoky bats, are represented by 2 monotypic genera, *Furipterus* and *Amorphochilus,* distributed in South America (Corbet and Hill, 1986). Fain (1976) described *Ewingana amorphochilus,* male only, as a parasite of *Amorphochilus schnabli.* He characterized the species as being distinct from other members of the genus *Ewingana* having a spiniform anterodorsal seta on trochanter I. The mites taken from *Amorphochilus* reveal that a new genus should be erected for them and for *E. amorphochilus.*

Furipterobia n. gen.

Gnathosoma broad. Idiosoma elongate; *vi* minute in male, expanded and striated in female; *ve, sc e,* and l_1 of male, 4 pairs of propodosomals and l_1 of female with gourd-form striated part; hysterosomal setae consisting of d_1 on genital shield, d_2, d_{4-5}, l_1, and l_{3-5} in male, d_{1-5} (complete) and l_{1-5} (complete) in female; *cx*I–IV 2-3-0-1. Genitalia: Male genital shield bearing more than 5 pairs of genital setae; vulvar lobes well developed, slightly overlapping distally and genital setae g_1 lacking in female. Legs: Leg I 4-segmented; setiform striated formation ventrally on tibio-tarsus I; all setae on tibio-tarsus I simple; anterodorsal seta on trochanter I peglike and striated; claw formula 0-2-1-1, with second claw on leg II being inferior in size to first one; setal formula for coxal regions I–IV 2-3-0-1. Solenidia



FIGURES 11–13. *Furipterobia chileensis* n. gen., n. sp., male. **11.** Dorsum. **12.** Venter. **13.** Genital shield.

on legs I and II as in Figure 11; a long and short and thin solenidion on tibio-tarsus I.

Type species: Ewingana amorphochilus Fain, 1976: 27.

Type host: Amorphochilus schnabli.

Furipterobia chileensis n. sp.
(Figs. 11–15)

Male (Figs. 11–13): Gnathosoma short dorsally. Body 250 (holotype)–225 (paratype) long by 125–115 wide. Hysterosomal setae d_2 slightly thickened and striated, with minute tail; l_1 and d_{4-5} fine and setiform; ventral setae ic_3 thickened, striated, and distinctly longer than other *ic* and *cx* series of setae. Genital shield situated posterior to basal level of *sc e,* and bearing 7 pairs of genital setae; longest genital setae measuring 55–53. Chaetotaxy on legs II–IV: Trochanters 3-2-2; femora 5-2-2; genua 7-5-5; tibiae 6-6-6; tarsi 6-6-6.

Female (Figs. 14, 15): Gnathosoma long dorsally. Body 315 (allotype)–290 (paratype) long by 165–155 wide. Dorsal setae d_{1-3} and l_{2-3} basally striated and with thin tail; only d_5 and l_4 simple setiform. Other structure and leg chaetotaxy as in male.

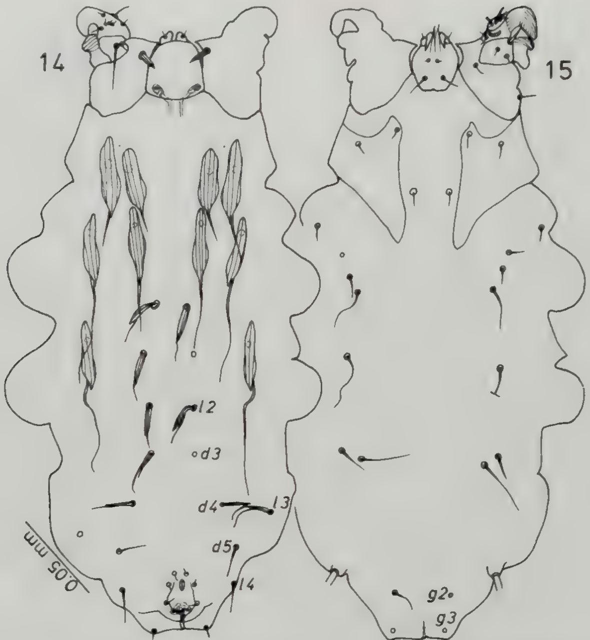
Material examined: Holotype male, allotype female, a male and female paratype and a male and female *ex Amorphochilus schnabli,* Cuya, Chile, date uncertain (USNM 391782). The holotype male of *Ewingana amorphochilus* Fain (BM 1980·20·217) from Peruvian *A. schnabli,* and a female of *Furipterobia* sp. *ex Furipterus harrens,* Kartabo, British Guiana (AM 142902–4) were also examined for comparison.

The types of *F. chileensis* n. sp. are deposited in the U.S. National Museum of Natural History Collection; other specimens are in the collection of the author.

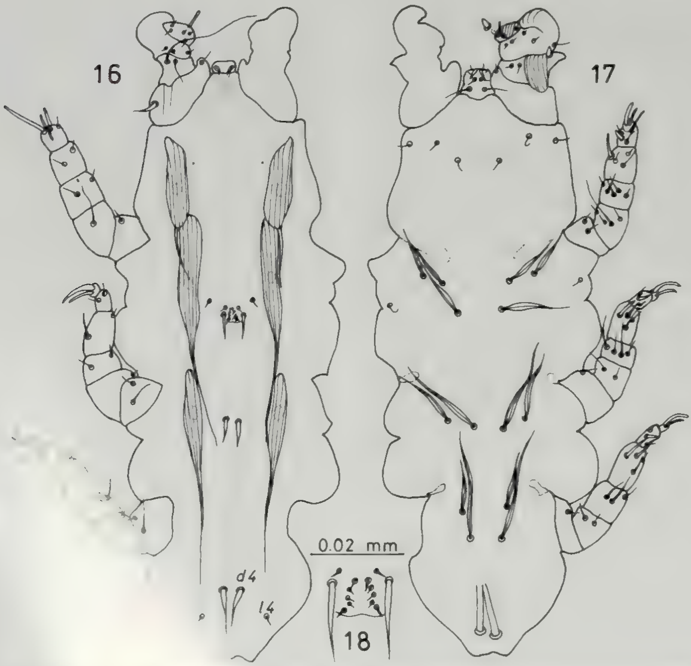
Remarks: Although the new mite was found on *A. schnabli,* the type host of *E. amorphochilus,* its male is easily separable from that of *E. amorphochilus* by differences in the lengths of the *ic* series of setae and in the arrangement of the first row of genital setae. The thickened and striated ventral setae ic_3 is specific to the male of the new species. Study of the female reveals that the new mite belongs in a genus other than *Ewingana* Radford as it has well-developed vulvar lobes instead of strongly modified genital setae g_7 of the genus *Ewingana.* Thus, the new genus *Furipterobia* n. gen. is erected for the new mite, *F. chileensis* n. sp., and *E. amophochilus* Fain.

As recorded above, Chilean and Peruvian *Amorphochilus* yielded different species of mites of the genus *Furipterobia,* suggesting that the bats named the same in the 2 localities are not conspecific, and should be reexamined systematically.

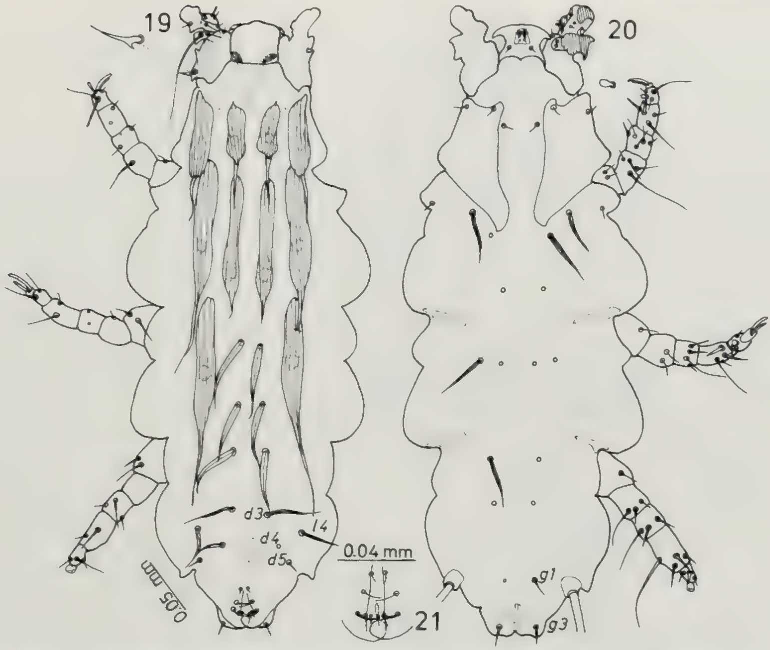
The family Thyropteridae, or New World sucker-footed bats, include 1 genus and 2 species distributed in southern Mexico, Peru, and southeastern Brazil (Corbet and Hill, 1986). They harbor a myobiid of the genus *Thyromyobia* which is defined largely on the female. Both sexes of a new mite of this genus were found in the present study and are described below, supplementing



FIGURES 14, 15. *Furipterobia chileensis* n. gen., n. sp., female. **14.** Dorsum. **15.** Venter.



FIGURES 16–18 *lor* n. sp., male. **16.** Dorsum. **17.** Venter. **18.** Genital shield.



FIGURES 19–21. *Thyromyobia tricolor* n. sp., female. **19.** Dorsum. **20.** Venter. **21.** Genito-anal region.

the generic definition with new information on the male.

Thyromyobia Fain, 1976

Acanthophthirius (*Thyromyobia*) Fain, 1976: 15.
Thyromyobia Fain, 1978b: 207.

Gnathosoma rectangular dorsally. Idiosoma elongate; *vi* not seen in male, expanded and striated in female; *ve, sc e,* and l_1 in male and 4 pairs of propodosomal setae and l_1 in female strongly expanded and striated; hysterosomal setae consisting of d_{1-2}, d_4, l_1, l_4, and l_5 in male and of d_{1-5} (complete), l_{1-2}, l_4, and l_5 in female; ventral setae on coxal regions II–IV thickened and striated. Genitalia: Male genital shield weak, bearing more than 5 pairs of minute setae and d_1; in female, vulvar lobes developed, genital setae g_2 lacking. Legs: Leg I 4-segmented, ventrally with striated formation and a conical seta on tibio-tarsus; claw formula 0(2)-2-2-2; setal formula for coxal regions I–IV 2-3-1-1. Nymphal legs I bilaterally symmetrical. Solenidia on legs I and II as in Figures 16 and 17.

Type species: Thyromyobia peruvianus Fain, 1976: 15.

Type host: Thyroptera discifera (from Peru, Fain, 1976).

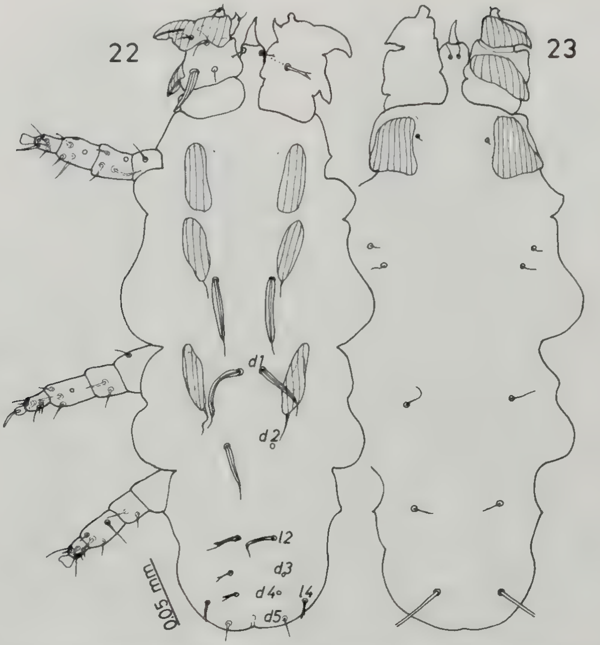
Thyromyobia tricolor n. sp.
(Figs. 16–27)

Male (Figs. 16–18): Holotype in skin of tritonymph 360 long by 132 wide. Setae *sc i* minute, situated anterolaterad from genital shield; d_1, d_2, and d_4 gaining thickness in this order. Genital shield bearing 6 pairs of minute genital setae and d_1. Posterolateral seta on trochanter I thick; both setae dorsally on femur I fine, with external one being long; spiniform seta each ventrally on tarsi II–IV; no leg seta long. Chaetotaxy on legs II–IV: Trochanters 3-3-3; femora 5-3-3; genua 7-6-6; tibiae 6-6-6; tarsi 6-6-6. Second claw on legs II–IV inferior in length and thickness to first one.

Female (Figs. 19–21): Allotype 532 long by 220 wide. Maximum width of setae *ve, vi, sc e, sc i,* and l_1, 20, 18, 20, 18, and 20, respectively; d_1, d_2, and l_2 the same in nature to each other; d_3, d_4, and l_4 thinner than preceding ones; d_5 fine and setiform. Genital seta g_7 being the thickest and *ae* the longest among genito-anal setae; spermatheca small (Fig. 19). Ventral setae ic_2, ic_3, and ic_4 missing, but structure probably as in male. Posterolateral seta on trochanter I and a ventral seta each on tarsi II and III modified as in Figures 19 and 20. Other structures essentially as in male.

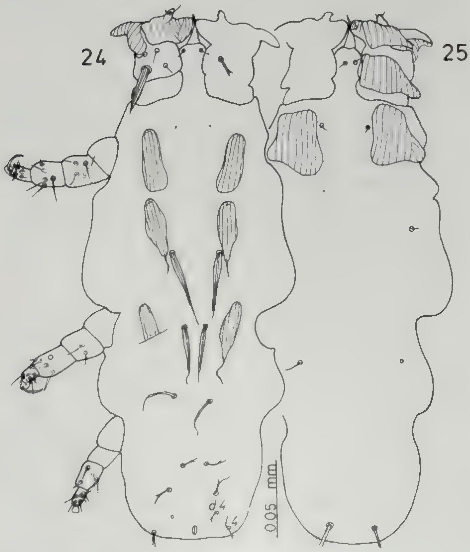
Deutonymph (Figs. 22, 23): Body 420–253 long by 175–115 wide (2 specimens). Dorsal setae *ve* oval, striated and lacking tails; *vi* lacking; *sc e* oval and striated at base, with thin tail; *sc i* situated on posterior level to bases of *sc e*, thickened and with short tails; l_1 and d_1 the same in nature to *sc e* and *sc i*, respectively; d_2 the same in nature to d_1; d_{3-5}, l_2, and l_4 present on opisthosoma, only d_5 setiform. Ventral setation on coxal regions I–IV 2(1 striated)-2-1-1. Dorsolateral gnathosomal seta forked at tip. Legs I as depicted in Figures 22 and 23; leg setal formula on trochanters II–IV 1-1-0, femuro-genua 4-2-2; tibiae 5-4-4; tarsi 6-6-6.

Protonymph (Figs. 24, 25): Body 335 long by 140 wide. Setation and setal nature as in deutonymph but



FIGURES 22, 23. *Thyromyobia tricolor* n. sp., deutonymph. **22.** Dorsum. **23.** Venter.

lacking d_5 dorsally and ic_4 and *cx*II ventrally. Lacking lateral seta or striated formation on femuro-genu I. Setal formula on trochanters II–IV 0-0-0; femuro-genua 4-2-0; tibiae 5-5-4; tarsi 6-6-6.



FIGURES 24, 25. *Thyromyobia tricolor* n. sp., protonymph. **24.** Dorsum. **23.** Venter.

Larva (Figs. 26, 27): Body 280 long by 103 wide. Propodosomal setae the same in number and nature to those of nymphal stages; hysterosomal setae consisting of 5 pairs (Fig. 26); ic_1 and large striations on coxal regions I. Legs I as in Figures 26 and 27. Setal formula on trochanter II–III 0-0; femuro-genua 2-0; tibiae 5-4; tarsi 6-6.

Material examined: Holotype male, allotype female, 2 deutonymphs, a protonymph, and a larva *ex Thyroptera tricolor albiventer,* Magdalena, Colombia, date uncertain (USNM 281197–200, 281202).

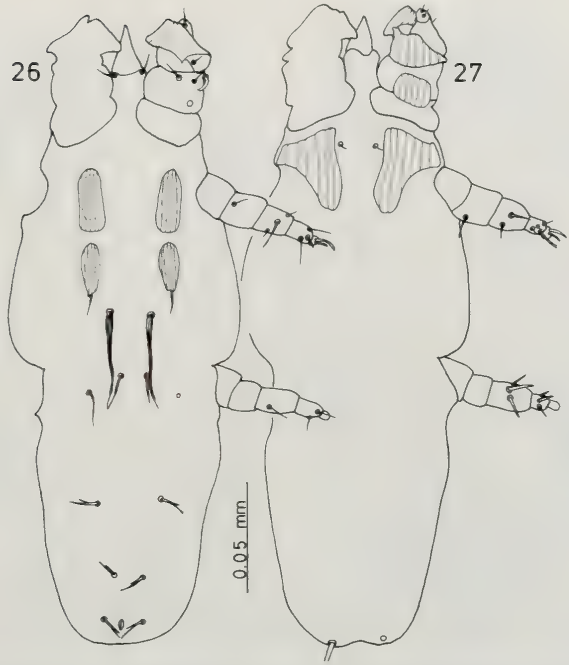
The holotype and allotype are deposited in the U.S. National Museum of Natural History Collection, and other specimens are in the collection of the author.

Remarks: Fain (1976) originally described *Acanthophthirius* (*Thyromyobia*) *peruvianus* on the basis of the female holotype and its tritonymphal pelt. Later (Fain, 1978b), he elevated *Thyromyobia* to generic status.

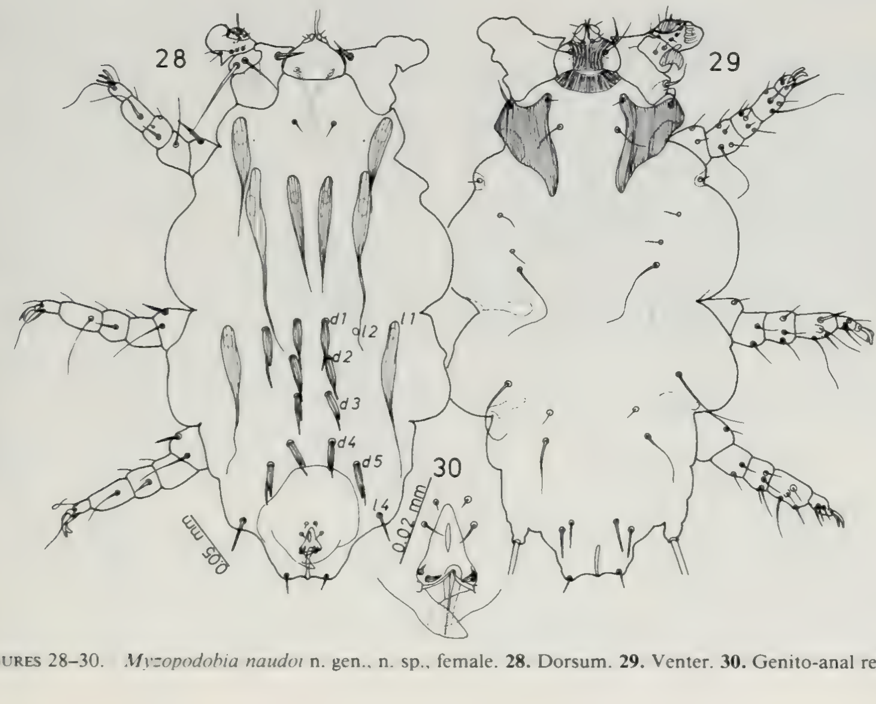
The new mite taken from *Thyroptera tricolor* is separable from *T. peruvianus* in the female by the broad gnathosoma and by lacking terminal claws on tibiotarsus I. Other structures, setation, and setal shape are essentially the same in the females of both species. Setae d_5 were lacking in the original figure of *A.* (*T.*) *peruvianus* (Fain, 1976, fig. 16), however, examination of the holotype indicates they are present.

Dimorphic deutonymphs probably exist in *T. tricolor,* judging from the difference in size as described above.

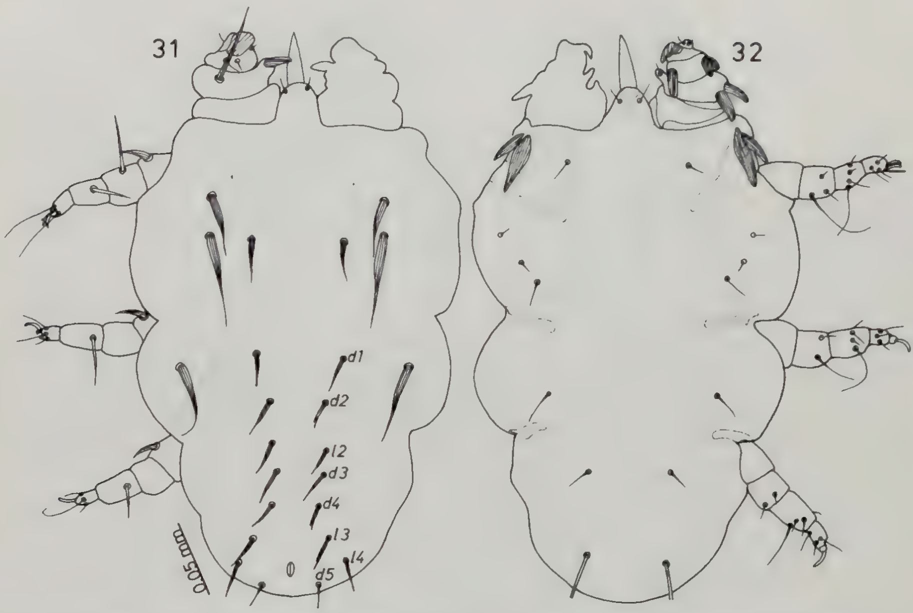
The family Myzopodidae is represented by only a single genus and 1 species, *Myzopoda aurita,*



FIGURES 26, 27. *Thyromyobia tricolor* n. sp., larva. **26.** Dorsum. **27.** Venter.



FIGURES 28–30. *Myzopodobia naudoi* n. gen., n. sp., female. **28.** Dorsum. **29.** Venter. **30.** Genito-anal region.



FIGURES 31, 32. *Myzopodobia naudoi* n. gen., n. sp., tritonymph. **31.** Dorsum. **32.** Venter.

the sucker-footed bat. A new myobiid genus was found to be associated with this species in the present study.

Myzopodobia n. gen.

Female: Gnathosoma bell-shaped; a pair of long and fine hypostomal setae. Idiosoma oval, with a pair of posterolateral projections including bases of l_5; *vi* fine and short, hysterosomal setae consisting of d_{1-5} (complete), l_{1-2}, and l_{4-5}; l_2 situated exteriad from *d* series of setae. Genitalia: Vulvar lobes well developed, overlapping distally; genital setae complete (g_{1-7}), with g_{1-2} being strong. Legs: Leg I 4-segmented; conspicuous striated structure ventrally on tibio-tarsus I; all setae on tibio-tarsus I simple; anterodorsal seta on trochanters I–IV spiniform; claw formula 0-2-2-2, with paired claws subequal to each other; setal formula for coxal regions I–IV 2-3-0-1. Solenidia on legs I and II as in Figures 28 and 29. Nymphs: Legs I bilaterally symmetrical.

Type species: Myzopodobia naudoi n. sp.

Type host: Myzopoda aurita.

Myzopodobia naudoi n. sp.
(Figs. 28–34)

Female (Figs. 28–30): Holotype 440 long by 220 wide. Gnathosoma with fine striation ventrally. Dorsal setae *ve* elongate oval at striated part; *sc e* and l_1 the same in nature to each other, with concave margins basally; *sc i* thickened, striated, and tapering; d_1–d_5 stout, 40, 32, 30, 28, and 30 long, respectively; l_2 the same in nature to *d* series of setae, 48 long; l_4 setiform, with blunt tip and 30 long. Sclerites on coxal regions I striated finely. Ventral chaetotaxy as in Figure 29. Chaetotaxy on legs II–IV: Trochanters 3-3-3; femora 5-3-3; genua 7-6-6; tibiae 6-6-6; tarsi 6-6-6.

Tritonymph (Figs. 31, 32): Idiosoma oval; legs relatively short. Body 330 long by 215 wide. Propodosomal setae consisting of *ve, sc e,* and *sc i,* and *vi* lacking; hysterosomal setae complete, d_{1-5} and l_{1-5}. Coxal setae 2(thickened and striated)-1-0-0; ventral setae on femur I and genu I thickened and striated; anterodorsal seta on trochanters II–IV spiniform and striated. Chaetotaxy on legs II–IV: Trochanters 1-1-1; femuro-genua 4-2-2; tibiae 5-4-4: tarsi 6-6-6. Claw formula 0-2-1-1.

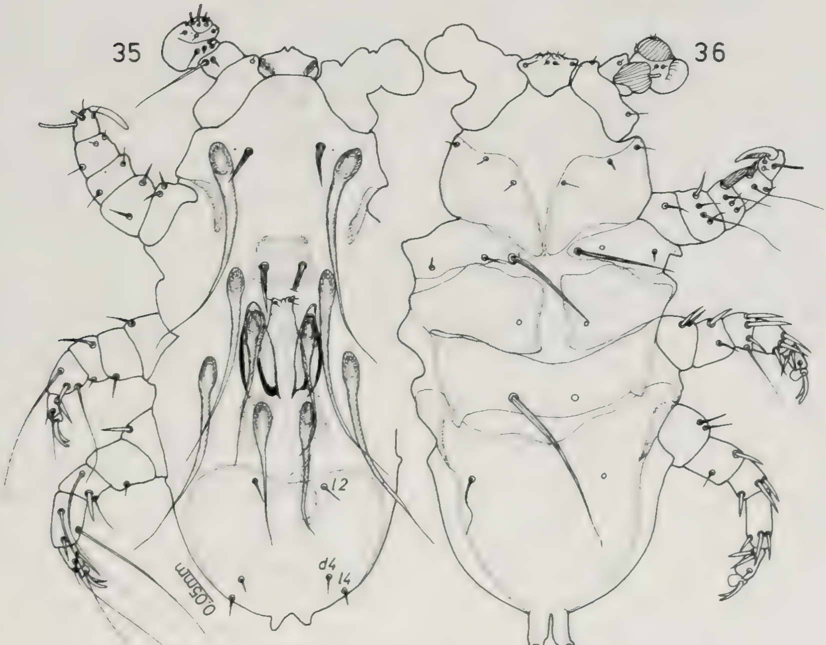
Deutonymph (Figs. 33, 34): Structures of gnathosoma, idiosoma, and legs and nature of setae on all parts almost the same to those in tritonymph, but smaller in size and setal number as compared with tritonymph. Body 290 long by 190 wide. Hysterosomal setae lacking l_4; coxal setae 1-1-0-0. Chaetotaxy on legs II–IV: Trochanters II–IV 1-1-0; femuro-genua 4-2-1; tibiae 5-4-4; tarsi 6-6-6. Claw formula 0-2-1-1.

Material examined: Holotype female *ex Myzopoda aurita,* Malunga Cotew, Madagascar, 12 June 1940 (in MNHN); a tritonymph and deutonymph *ex M. aurita,* Mahambo, Madagascar, 9 May 1876 (RMNH 26117). The holotype is deposited in the collection of MNHN and nymphal specimens are in the collection of RMNH (RMNH P 2065—deutonymph; RMNH P 2066—tritonymph).

Remarks: The posterolateral idiosomal outline, position of setae l_2, and nature of the hysterosomal setae, d_{1-5} and l_2, of the female mite of the genus *Myzopodobia* n. gen. are different from those of all the other chiropteran myobiid genera.

It is a great pleasure to name the new mite after Dr. Michael Henri Naudo, Museum National d'Histoire

FIGURES 33, 34. *Myzopodobia naudoi* n. gen., n. sp. deutonymph. **33.** Dorsum. **34.** Venter.



FIGURES 35, 36. *Mystacobia hirusta* Fain, male. **35.** Dorsum. **36.** Venter.

Naturelle de Paris, who helped the author in numerous ways during his stay in Paris.

The family Mystacinidae, or New Zealand short-tailed bats, consist of 1 genus and 2 species. A strongly specialized mite of the genus *Mystacobia* has been described on the basis of female and nymphal specimens (Fain, 1972b). A male specimen judged to belong to the species *M. hirsuta* was found in the present study.

Genus *Mystacobia* Fain, 1972

Mystacobia Fain, 1972b: 153; Fain, 1978c: 196.

Female: Coxal shields present ventrally. Leg I 5-segmented, with tibia entirely striated ventrally. Idiosomal setae consisting of 4 pairs of propodosomal setae, $d_{1-2,4-5}$ and l_{1-5} (complete); 4 pairs of propodosmal setae, d_{1-2} and l_1 thickened and striated; other hysterosomal setae simple setiform. Claw formula 0-2-2-2, with paired claws on legs II–IV subequal in size to each other. Vulvar lobes well developed. Coxal setae 2-3-0-0. Chaetotaxy on legs II–IV: Trochanters 3-3-3; femora 5-3-3; genua 7-6-6; tibiae 6-6-6; tarsi 7-6-6 (Fain, 1972b, 1978a). Male: Gnathosoma broad and short. Idiosoma elongate; dorsal sclerites bearing setae prominent as ventral ones; posterior projections carrying terminal setae l_5; hysterosomal setae consisting of $d_{1-2,4}$ and $l_{1-2,4-5}$. Legs II–IV stout, each bearing some long setae; a single, strong claw on leg II. Solenidia on legs I and II as in Figure 35.

Type species: Mystacobia hirsuta Fain, 1972b: 153.
Type host: Mystacina tuberculata.

Mystacobia hirsuta Fain
(Figs. 35, 36)

Male (Figs. 35, 36): Plesiotype 720 long from top of gnathosoma to base of l_5 and 315 wide. Idiosoma elongate; *vi* stout, striated, and setiform, 65 long; *sc i* the same in nature to *vi*, 65 long; *sc i* the same in nature to *vi*, on sclerite, 55 long; *ve*, *sc e*, and l_1 the same in nature to one another, long, basally inflated, and tapering and striated as in Figure 35; d_1 and d_2 the same in nature to but much shorter than *ve*, *sc e*, and l_1, on sclerites, respectively; d_4, l_2, and l_4 short and setiform, on a large opisthosomal sclerite. Paired sclerites dorsally close to trochanters II and ventrally aside to trochanters IV. Ventral sclerites and setation as in female, though ic_2 missing. Genital shield situated between setae d_1, large, flask-shaped, bearing 2 pairs of minute setae anterolaterally, and joined to sclerites flanking setae d_1 posterolaterally; penis stout and long. Legs I as in female; anteroventral seta on genu and tibia II thickened and striated; 1 or 2 long dorsal setae on genu IV, tibiae III and IV, and tarsi III and IV.

Material examined: Plesiotype male *ex Mystacina tuberculata*, New Zealand, November 1967 (AM 214243). The plesiotype is deposited in the collection of the Department of Entomology, American Museum of Natural History.

Remarks: Both sexes of *M. hirsuta* show a hypersclеritization on idiosomal dorsum and venter, which is not shared by any genus of chiropteran Myobiidae. *Mystacobia* is, however, close to *Acanthophthirius* parasitic on bats of the family Vespertilionidae in having 5-segmented leg I, 2 subequal claws each on legs II–IV, and, in the female, well-developed vulvar lobes.

DISCUSSION

The chiropteran Myobiidae consist of 22 genera, including the 3 new genera specific to minor host families and exclusive of *Rhynomyobia* (Fain, 1978b), which should be relegated to a subgenus of the genus *Acanthophthirius* as was suggested originally by Fain (1973a). Sixteen genera are represented by larger elongate mites, and 6 genera comprise smaller rounded forms. As in the insectivoran Myobiidae (Uchikawa, 1985c), a set of both larger- and smaller-sized genera are expected to be members of a given chiropteran family. However, smaller-sized genera have not been found in many host families, whereas the larger-sized genera are known from all families other than Rhinopomatidae, Craseonycteridae, Megadermatidae, and Noctilionidae (Table I).

Most of the myobiid genera are well defined in both sexes, and their validity is confirmed in the present study. Examination of the type of *Phyllostomyobia* Fain (1973a) in the British Museum (Natural History) (BM 1976·260) and of *Ioannela* Dusbábek and Lukoschus (1973) in Rijksmuseum van Natuurlijke Histoire reveals, however, that these 2 genera are congeneric. It is left to the originators of these 2 genera to determine which has priority.

Myobiid mites exhibit a high degree of host specificity and each myobiid genus is said to be restricted to only a single family or subfamily of hosts (Fain, 1979). This might be the case for genera represented by larger elongate forms in the chiropteran Myobiidae (Table I). There are, however, 2 examples discordant to the above trend in host–parasite relationships in Table I. First, members of the host family Emballonuridae are associated not with a single myobiid genus but with 2 allopatric genera. It is necessary to discuss with chiropterologists whether this is an exceptional case in host–parasite relationships or if it means that the Old and New World Emballonuridae harboring the genera *Ugandobia* and *Expletobia*, respectively, belong to different families. Second, contrary to Fain's (1979) hypothesis, the 3 host families Phyllostomatidae, Mormoopidae, and Desmodontidae share a single myobiid genus, *Eudusbabekia*. Recently, chiropterologists (Honacki et al., 1982; Corbet and Hill, 1986) relegated the former Desmodontidae to a

Table I. *Chiropteran families and myobiid genera, with key morphology for the mite genera.*

Host family	Larger-sized myobiid genus	Smaller-sized myobiid genus
Pteropodidae	*Binuncus* Radford. *Pteropimyobia* Fain Group 1: 4(2-2-2)* Group 1: 4(2-2-2)	–
Rhinopomatidae	–	*Hipposiderobia* Dusbábek‡ Group 1: 5(2-2-2)
Emballonuridae	*Ugandobia* Dusbábek (Old World) Group 1: 4(2-1-1) *Expletobia* Dusbábek (New World) Group 3: 4(2-1-1)	–
Craseonycteridae	–	–
Nycteridae	*Nycterimyobia* Fain Group 1: 4(2-2-2)	–
Megadermatidae	–	–
Rhinolophidae	*Neomyobia* Radford Group 3: 4(2-2-2) (*Rhinomyobia* Fain)† Group 3: 5(2-2-2)	–
Hipposideridae	*Metabinuncus* Fain Group 3: 4(2-2-2)	*Hipposiderobia* *Binunculoides* Fain Group 1: 4(2-2-2) *Triaenomyobia* Fain Group 1: 4(2-2-2)
Noctilionidae	–	–
Phyllostomatidae (including Mormoopidae and Desmodontidae)	*Eudusbabekia* Jameson Group 3: 4(1-1-1) or (1-2-2)	*Phyllostomyobia* Fain or *Ioannela* Dusbábek and Lukoschus Group 3: 4(2-2-2)
Natalidae	*Natalimyobia n. gen.* Group 3: 4(2-1-1)	–
Furipteridae	*Furipterobia n. gen.* Group 3: 4(2-1-1)	–
Thyropteridae	*Thyromyobia* Fain Group 3: 4(2-2-2)	*Pteracarus* Jameson et Chow Group 1: 5(2-2-2)
Myzopodidae	*Myzopodobia* n. gen. Group 3: 4(2-2-2)	–
Miniopteridae	*Calcarmyobia* Radford Group 3: 4(2-2-2)	*Pteracarus*
Vespertilionidae	*Acanthophthirius* Perkins Group 3: 5(2-2-2)	*Pteracarus*
Mystancinidae	*Mystacobia* Fain Group 3: 5(2-2-2)	–
Molossidae	*Ewingana* (*Ewingana*) Radford Group 2: 4(2-1-1) *Ewingana* (*Doreyana*) Dusbábek Group 2: 4(2-2-2)	*Schizomyobia* Fain Group 2: 4(2-2-2)

* Leg formation formula: The vulva (Group 1—naked; Group 2—protected by modified genital setae g_7; Group 3—protected by vulvar lobes), number of segments of leg I and, in parentheses, claws on legs II–IV are presented in this order.

† The monotypic, least known genus, which should be relegated to a subgenus of *Acanthophthirius*.

‡ The occurrence of this genus will be detailed elsewhere.

subfamily of the family Phyllostomatidae, and thus the host–parasite relationship partly agrees with Fain's (1979) assumption. However, chiropterologists regard the family Mormoopidae as valid, requiring further discussions between acarologists and chiropterologists.

It is difficult to deduce a clear trend in host–parasite relationships from a total of only 6 smaller-sized genera. The genus *Pteracarus* is shared by the families Vespertilionidae, Miniopteridae, and Thyropteridae (Table I). Mites of the genus *Pteracarus*, parasitic on the family Miniopteridae, were studied in detail and only 2 species were recorded from various bats that yielded 25 species and subspecies of mites of the genus *Calcarmyobia* (Uchikawa, 1985a, 1985b). Speciation of *Pteracarus* is much less when compared with its partner larger-sized genus. The same trend is also observed in the genera *Phyllostomyobia* or *Ioannela* and *Schizomyobia*. It is reasonable to presume that smaller-sized genera are likely shared between allied host families.

TABLE II. *Grouping of allied myobiid genera according to female genitalia.*

Group	Key characteristics	Larger-sized genera (host family)	Smaller-sized genera (host family)
Group 1	Vulva naked	*Binuncus* (Pteropodidae) *Pteropimyobia* (Pteropodidae) *Nycterimyobia* (Nycteridae) *Ugandobia* (Old World Emballonuridae)	*Pteracarus* (Vespertilionidae, Miniopteridae, Thyropteridae) *Hipposiderobia* (Hipposideridae, Rhinopomatidae) *Triaenomyobia* (Hipposideridae) *Binunculoides* (Hipposideridae)
Group 2	Vulva protected by specialized g_7	*Ewingana* (Molossidae)	*Schizomyobia* (Molossidae)
Group 3	Vulva protected by vulvar lobes	*Acanthophthirius* (Vespertilonidae) *Mystacobia* (Mystacinidae) *Calcarmyobia* (Miniopteridae) *Thyromyobia* (Thyropteridae) *Metabinuncus* (Hipposideridae) *Neomyobia* (Rhinolophidae) *Myzopodobia* (Myzopodidae) *Natalimyobia* (Natalidae) *Furipterobia* (Furipteridae) *Expletobia* (New World Emballonuridae) *Eudusbabekia* (Phyllostomatidae)	*Phyllostomyobia* or *Ioannela* (Phyllostomatidae)

The host relations of the genera *Hipposiderobia, Triaenomyobia,* and *Binunculoides* are, however, not in accordance with this assumption (Table I), if systematic uniformity of the present Hipposideridae is true.

As mentioned above, some problems in the systematics of both myobiid mites and their chiropteran hosts may be seen in light of host–parasite relationships. However, other concepts and procedures are necessary to relate parasitological data phylogenetically and to host phylogeny.

The basic principle of parallel phylogeny between parasite and host groups has been confirmed in a considerable number of cases and is underlined in the first symposium on host specificity among parasites of vertebrates (Mayr, 1957). Accordingly, when a phylogeny of chiropteran Myobiidae is established, it will serve to complement our knowledge of phylogenetic relationships among host groups. Jameson (1955) pointed out that structures of legs I, number of the claws on legs II–IV, and chaetotaxy on the idiosoma are indicative of phylogenetic position of a given myobiid genus. Dusbábek (1969a) and Fain (1975, 1979, 1982) largely followed Jameson, although Dusbábek also examined the structure of the female genitalia carefully. The fusion of some segments of leg I and reduction or disappearance of the second claw on legs II–IV are interesting evolutionary changes, but might be adaptive or secondary. I (Uchikawa and Harada, 1981) found that minor but distinctive differences in the male genitalia are more suggestive of intrageneric phylogenetic relationships among myobiid mites than are discrepancies in the morphology of legs and idiosomal chaetotaxy. Although phylogenies of the genera *Calcarmyobia* (Uchikawa, 1985a) and *Binuncus* and *Pteropimyobia* (Uchikawa, 1985b) were proposed on the basis of structure of the male genitalia, this structure is too variable to be used to deduce intergeneric relationships among the chiropteran Myobiidae, and more conservative characteristics are necessary. A comparative study of all known genera of the chiropteran Myobiidae suggests that the female genitalia might be of value in determining relationships.

The female genitalia consist of the vulva and 7 pairs of genital setae and are fundamentally the same in all myobiids parasitic on marsupials, insectivores, chiropterans, and rodents (Fain, 1973b), suggesting a monophylogenetic origin of the family. The vulva is naked in some mite genera (Fig. 5), while it is protected by modified genital setae g_7 or by vulvar lobes (=vulvar valves in Dusbábek, 1969a, 1969b) (Figs. 10, 14, 21, 30). It is reasonable to regard the protected vulva as being more specialized or evolved than a naked one.

The 16 genera represented by larger elongate forms of the chiropteran Myobiidae in Table I are subdivided into 3 different groups according to the structure of the female genitalia (Table II). The genera *Metabinuncus* and *Calcarmyobia* were thought not to have vulvar lobes (Dusbábek, 1969b), but vestigial or primitive lobes are discernible in *Metabinuncus,* and *Calcarmyobia* bears large and membranous lobes that are rather difficult to observe.

The 4 genera assigned to Group 1 are thought

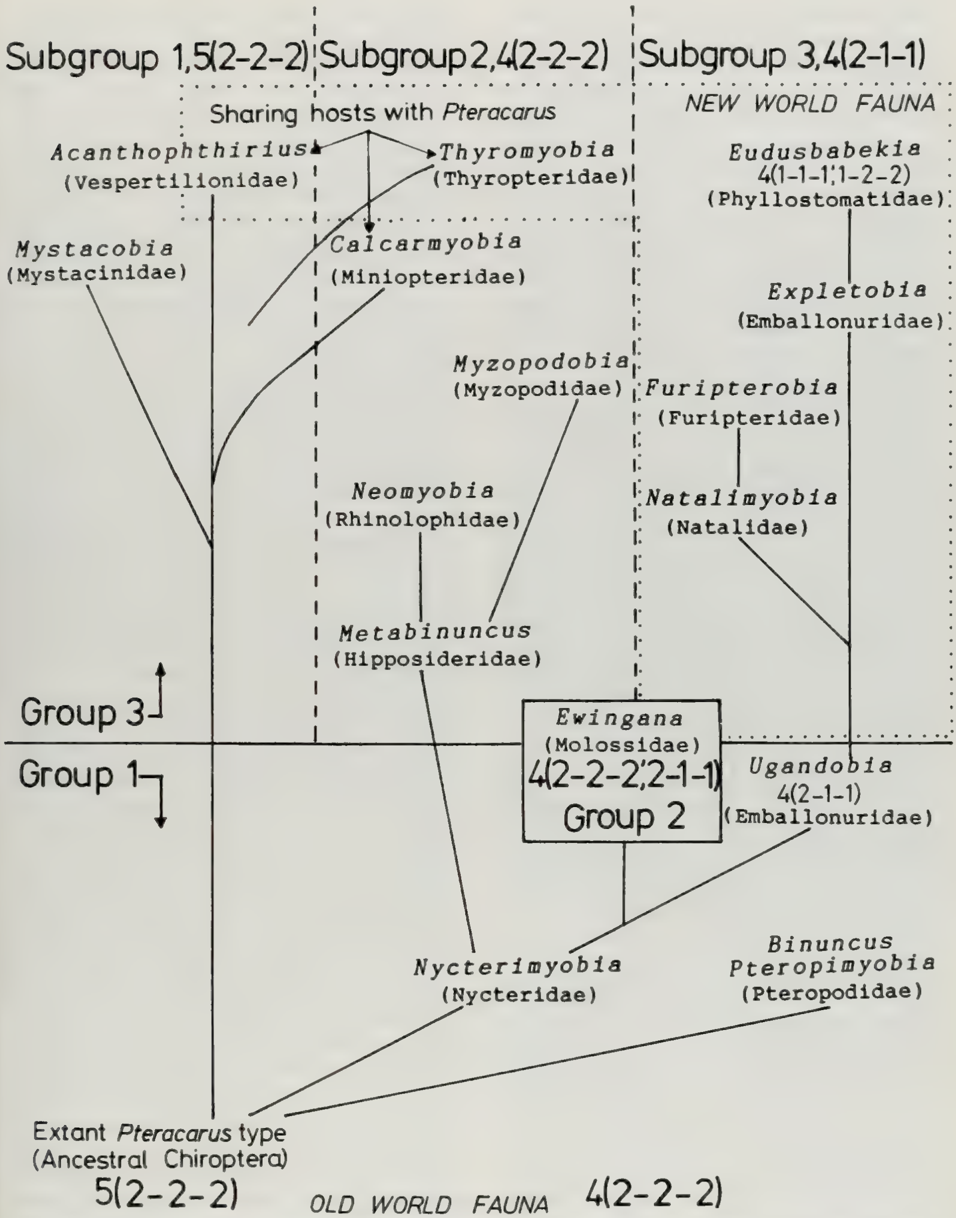


FIGURE 37. Proposed phylogeny of chiropteran Myobiidae, suggestive of that of host Chiroptera shown in parentheses. Group 1–Group 3 and leg formation formula as in Table I.

to be the most primitive among the chiropteran Myobiidae. The genus *Ewingana* (Group 2) has probably evolved from an ancestor of an extant *Nycterimyobia* type together with an extant *Ugandobia* type that includes some forms with female genitalia of an intermediate type of Groups 1 and 2. The 11 divergent genera in Group 3 are thought to be most evolved. Although there are

primitive and developed vulvar lobes suggesting some evolutionary lines, it is difficult to relate closer genera in a given group to one another based merely on the formation of female genitalia. Therefore, such secondary characteristics as reduction in number of leg segments and claws and specializaiton of idiosomal and leg chaetotaxy are introduced to deduce phylogenetic relationships from genera assigned to each group. Ancestral chiropteran myobiids might be of an extant *Pteracarus* type with naked vulva, simple setiform setae on the idiosoma and legs, and a leg formation formula of 5(2-2-2), indicating number of segments of leg I and numbers of claws on legs II–IV in parentheses. However, all of the most primitive genera in Group 1 bear a 4-segmented leg I, suggesting that the reduction in number of segments occurred first and offshoots of the 4 genera from ancestral stock followed. Among the 4 genera, *Nycterimyobia* is thought to be the most primitive because of its simple setae on the idiosoma and leg I and a 2-2-2 claw formula; *Binuncus* and *Probinuncus* seem close to each other phylogenetically despite a difference in general morphology (Uchikawa, 1986b). However, these genera bear specialized setae and striated formation ventrally on the idiosoma and leg I, respectively, both of which are simple and primitive in *Nycterimyobia* and *Ugandobia*; and only *Ugandobia* shows an advanced claw formula of 2-1-1. On the basis of all of these characteristics, it is reasonable to presume that *Nycterimyobia, Binuncus,* and *Pteropimyobia* appeared first and that *Ugandobia* stemmed from *Nycterimyobia* as shown in Figure 37.

The genus *Ewingana* is the sole representative of Group 2, indicating the unique evolutionary direction of this genus. Some species of the genus show a 2-2-2 claw formula, while others exhibit 2-1-1. It is exceptional to observe 2 different claw formulae among congeneric species. Provided that the regression of a claw on leg III and IV occurred once and ceased without spreading over the whole ancestral *Ewingana,* the genus *Ewingana* might have branched from a *Nycterimyobia–Ugandobia* line, preceding establishment of *Ugandobia,* and which also bears a trace of developing genital seta g_7. All genera with a 2-1-1 claw formula are thus thought to be descendants of a *Ugandobia* type of myobiid.

The 11 genera relegated to Group 3 are divergent, but they consist of 3 subgroups defined by differences in number of segments of leg I and claws on legs II–IV as follows: Subgroup 1 with a leg formation formula of 5(2-2-2), Subgroup 2 with 4(2-2-2), and Subgroup 3 with 4(2-1-1), 4(1-1-1), and 4(1-2-2) (Fig. 37). Subgroup 1 includes *Acanthophthirius* and *Mystacobia.* These genera are likely the most primitive forms among the chiropteran Myobiidae because of their ancestral leg formation. However, genera in Group 3 should be regarded as having a higher evolutionary status, as mentioned above, and the ancestral characteristics of *Acanthophthirius* and *Mystacobia* are thought to have remained in myobiids derived directly from an original stock of the chiropteran Myobiidae.

Subgroup 2 includes *Metabinuncus, Neomyobia, Myzopodobia, Calcarmyobia,* and *Thyromyobia. Metabinuncus–Neomyobia* and *Metabinuncus–Myzopodobia* lines of evolutionary changes are presumed on the basis of degree of development in vulvar lobes. *Neomyobia* bears very primitive vulvar lobes and developed seta *vi* compared with *Myzopodobia.* When 2 major characters of 2 genera do not show the same proposed direction in evolutionary changes, then the genera should be regarded as being on different evolutionary lines. Accordingly, *Neomyobia* and *Myzopodobia* are thought to have diverged from *Metabinuncus* (Fig. 37). Concerning the relations between *Metabinuncus* and genera in Group 1, *Nycterimyobia* bears characteristics that are all more primitive than those of *Metabinuncus,* while *Binuncus* and *Pteropimyobia,* in spite of their primitive vulva, have more specialized setae on the ventral idiosoma than does *Metabinuncus.* It is thus better to relate *Metabinuncus* to *Nycterimyobia* than to *Binuncus* and *Pteropimyobia* (Fig. 37). On the other hand, *Calcarmyobia* and *Thyromyobia* have specialized setae somewhat similar to those of *Neomyobia* on the dorsal idiosoma. However, the vulvar lobes are more developed and idiosomal chaetotaxy is more primitive in *Calcarmyobia* and *Thyromyobia* than in *Neomyobia.* So it is difficult to relate the former 2 genera to any genus in Subgroup 2. A clue for assigning them proper positions is that the smaller-sized genus *Pteracarus* occurs concomitant with *Calcarmyobia* and *Thyromyobia* (Fain, 1978c) as well as *Acanthophthirius* in Group 1 on respective host families. From this, *Calcarmyobia* and *Thyromyobia* are thought to have shared an original stock with *Acanthophthirius,* although the relationship between the 2 genera is uncertain (Fig. 37).

Subgroup 3 consists of *Natalimyobia, Furi-*

pterobia, Expletobia, and *Eudusbabekia,* all belonging to the New World fauna. It is suggested above that these genera might have diversified from a stock similar to an extant *Ugandobia* type with a leg formation formula of 4(2-1-1). Two genera, *Expletobia* and *Eudusbabekia,* bear simple and ancestral setae *vi* as in *Ugandobia,* while the genera *Natalimyobia* and *Furipterobia* have modified *vi,* suggesting the presence of 2 evolutionary lines. *Eudusbabekia* is thought to be more evolved than *Expletobia,* since the former exhibits leg formation formulae of 4(1-1-1) or 4(1-2-2) instead of the latter's 4(2-1-1). In this case, the second claw on legs III and IV of *Eudusbabekia* disappears in a large majority of species and is rudimentary, with a claw formula of 1-2-2 in only a few species. Accordingly, the leg formation formula for *Eudusbabekia* is better represented by 4(1-1-1). On the other hand, *Natalimyobia* is more primitive than *Furipterobia* in having g_1 and in lacking striated formation ventrally on tibio-tarsus I (Fig. 37).

Thus, all of the 16 larger-sized genera are related to one another as summarized in Figure 37 based primarily on the female genitalia and secondarily on the leg formation and chaetotaxy of the idiosoma and legs. This proposed phylogeny of the chiropteran Myobiidae differs considerably from that presented by Dusbábek (1969a), reflecting a difference in adopting criteria for measuring phylogenetic relationships among genera.

Concerning the smaller-sized genera, slower evolutionary changes in the genito-anal region and in number of claws on legs II–IV are discernible compared with the partner larger-sized genera associated with respective host families (Table I). It is interesting to note that the genus *Schizomyobia* infesting the Molossidae is assigned to Group 2 together with its partner genus, *Ewingana.* Additional material for the smaller-sized myobiids should be assembled so data in Figure 37 could be supplemented with further information.

It is thought that parasites diversify not of themselves but under compulsion of diversification in host assemblage. This is the concept that supports the basic principle of parallel phylogeny between parasite and host and that enables parasitological data to be of value in the consideration of systematics and phylogeny of hosts. Many attempts have been made to use parasitological data in connection with host systematics and phylogeny. Parasites have often been examined only to confirm a hosts's systematic and/or phylogenetic status as proposed by mammalogists. However, it is more important to locate discrepancy between parasite and host systematics or phylogenies than to stress the agreement of information on parasites and their hosts, since such discrepancy may indicate problems in both parasite and host analyses. Following these considerations, a chiropteran phylogeny in parallel with myobiid phylogeny is proposed as follows (Fig. 37): (1) Ancestral Chiroptera might have arisen from an extant Vespertilionidae (including Mystacinidae, Miniopteridae, and Thyropteridae) type; (2) Megachiroptera and Nycteridae might have been derived from the ancestral stock first, with the latter being progenitors of Old World Emballonuridae, Molossidae, and Hipposideridae; (3) Old World Emballonuridae and Molossidae might have arisen at almost the same time, with the former being ancestors of the New World Emballonuridae, Phyllostomatidae (including Mormoopidae), Natalidae, and Furipteridae; (4) Hipposideridae might have been ancestors of Rhinolophidae and Myzopodidae; (5) the New World family Thyropteridae might have had relatives in an original stock of Chiroptera rather than in an extant Myzopodidae type of ancestor.

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ERRATUM: Please insert this corrected page on page 177 of this issue.

J Parasit. 74(1), 1988, pp. 177–179

RESEARCH NOTE . . .

Strongyloides stercoralis Infection in the Ferret

Richard A. Davidson, Department of Medicine, Box J277, University of Florida, Gainesville, Florida 32610

ABSTRACT: The ferret (*Mustela putorius furo*) was evaluated as an animal model for infection with human strains of *Strongyloides stercoralis.* Results indicate that such infections can be easily and reproducibly accomplished.

Human infection with *Strongyloides stercoralis* can be a serious cause of morbidity and mortality in immunocompromised individuals because of the tendency for disseminated infection (Igra-Siegman et al., 1981, Reviews of Infectious Diseases **3:** 397–407). The mechanism by which dissemination occurs has not been explained. Rodent animal models infected with species-specific strongyloides have not proven useful for research purposes because it has been impossible to induce hyperinfection and dissemination; for instance, in rats infected with *S. ratti,* the infection is cleared spontaneously, even in animals given corticosteroids (Genta and Ward, 1980, American Journal of Pathology **99:** 207–220). Two animal models for infection with *Strongyloides stercoralis* have been developed: dogs and patas monkeys (Grove and Northern, 1983, International Journal of Parasitology **13:** 483–490; Harper et al., 1984, American Journal of Tropical Medicine and Hygiene **33:** 431–450; Schad et al., 1984, Experimental Parasitology **57:** 287–296). Neither is an ideal candidate for an animal model because of expense in obtaining and maintaining these relatively large animals. We evaluated the ferret (*Mustela putorius furo*), a small carnivore that is easily maintained in the laboratory, as an animal model for infection with human strains of *Strongyloides stercoralis.*

Young adult male ferrets (6–12 mo old) were obtained from Marshall Research Animals (North Rose, New York). All animals had baseline stool examinations for parasites that were negative; all stool examinations were done by ethylacetate sedimentation. All *Strongyloides* larvae were acquired from an immunosuppressed dog that had been originally infected with larvae from a Southeast Asian.

First experiment: Seven ferrets were immunosuppressed with depo-methylprednisolone (Depo-Medrol, Upjohn). Information concerning the absorption rate of this drug after intramuscular injection in the ferret is unknown. Initial dosing was intended to approximate 8 mg/kg/day if given weekly, for a total initial dose of 56 mg/kg. Seven days after the first intramuscular injection a second dose was given. On the same day these 7 ferrets and 6 nonimmunosuppressed ferrets were infected with 3,500 filariform larvae of *Strongyloides stercoralis* that were mixed with penicillin and streptomycin. These larvae were injected subcutaneously in 3 aliquots of saline on the shaved abdomen.

The 6 nonimmunosuppressed ferrets developed slight induration on their abdominal walls in the area of injection. Over the next 6 wk, all feces remained negative for parasites (at 2–3-day intervals). The animals showed no obvious evidence of disease.

Within 2 or 3 days following infection, all immunosuppressed ferrets developed 0.5–2-cm necrotic ulcers at the site of injection. These areas were debrided and treated with daily application of debriding enzyme. On day 10 following infection, 2 animals had rhabditiform larvae of *Strongyloides stercoralis* in their stools. On day 15, 5 of the 7 animals had positive stools; 2 of these ferrets were losing weight and had decreased their food and water intake. One of these animals developed a paresis of the hind legs. By day 19, all animals had positive stools. White blood cells in the peripheral blood increased from a mean of 9,600 preinfection to 14,500 at 30 days postinfection; all animals had lymphopenia (mean 2%) and eosinopenia (none seen), reflecting the effect of the corticosteroid. The numbers of rhabditiform larvae found in the stools increased from approximately 3 per coverslip on day 12, to 16 per coverslip on day 23, to 45 per coverslip on day 35. All animals were necropsied by day 40; each had evidence of *Strongyloides stercoralis* infection in the submucosa of the duodenum (Fig. 1). Pathological review of the material revealed that 1 animal died of concurrent *Cryptosporidium* infection and fungal meningitis; 2 other animals had meningitis, with cultures positive for gram-negative organisms (*Proteus*

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and *Pseudomonas*), a frequent complication of dissemination of *Strongyloides stercoralis.* No larvae were seen in the central nervous system; Baermannization was not performed. A third animal was found to have a sterile cerebral vasculitis, another noted complication of dissemination (Wachter et al., 1984, Archives of Neurology **41**: 1213–1216). Two animals had evidence of muscle fiber degeneration, probably secondary to the effect of corticosteroids.

Second experiment: Because of the impression that the cause of death in the ferrets was due to the extent of immunosuppression and resultant opportunistic infection, 4 fresh animals were given 4 mg/kg and 2 animals 2 mg/kg of depo-methylprednisolone 3 days prior to infection (greater than a 10-fold decrease from the first experiment). Because of the severity of necrotic lesions when subcutaneously injected larvae were used, animals were infected by concentrating 5,000 filariform larvae in saline and applying the liquid to the shaved abdomen of anesthetized animals. Two animals that had been given 4 mg/kg died suddenly on days 7 and 9; by day 12 the 4 remaining animals had passed rhabditiform larvae in their feces. The maximum number of larvae per gram of feces ranged from 45 to 120. All 4 animals cleared their stools of larvae (on days 18, 29, 40, and 41) and remained parasite free in spite of 2 doses of 1 mg/kg of depo-methylprednisolone.

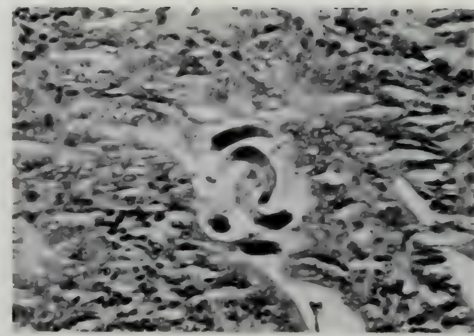

FIGURE 1. *Strongyloides stercoralis* in the duodenum of a ferret.

Third experiment: The above results suggested the need for a recurrent, higher dose of corticosteroid in order to produce persistent infection. Four ferrets were initially immunosuppressed with 8 mg/kg of depo-methylprednisolone. Nine days later they were infected with 5,000 filariform larvae percutaneously. By day

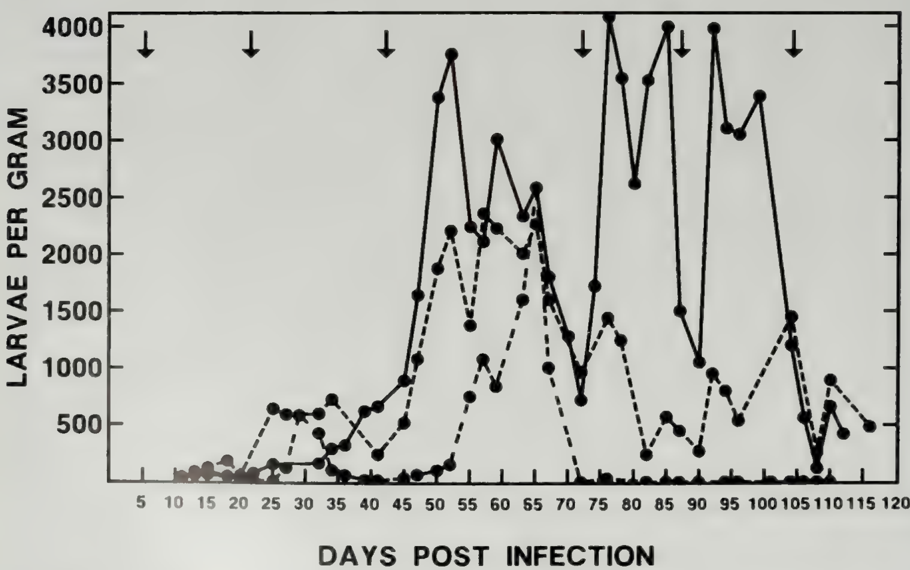

FIGURE 2. Course of infection in 3 ferrets infected with *Strongyloides stercoralis.* Each arrow represents a dose of depo-methylprednisolone, 8 mg/kg.

11 postinfection, all animals had positive stools for rhabditiform larvae. Recurrent doses of immunosuppression at the same dosage were given. On day 29, 1 ferret that had 1,612 rhabditiform larvae per gram of stool was sacrificed. The lungs were carefully washed with saline. There were no filariform larvae found in the lungs after they were Baermannized. The course of the infection in the remaining animals is described in Figure 2. The numbers of excreted larvae increased dramatically and quickly after most courses of immunosuppression. One animal cleared the infection after 76 days and 5 courses of immunosuppression; the other 2 had persistence of infection for 112 and 116 days, respectively, and 7 courses of immunosuppression. One ferret died at 116 days of bronchopneumonia. At necropsy there was no evidence of dissemination of infection.

After this study began, we became aware that one previous attempt to infect ferrets with a canine strain of *Strongyloides stercoralis* was unsuccessful (Schad, pers. comm.). However, the results of this study indicate that established infection of the ferret with human strains of *Strongyloides stercoralis* can be easily and reproducibly accomplished. Pathological evidence of dissemination of infection was lacking in spite of some suggestive findings, but relatively few animals were evaluated. It is possible that further investigation of *Strongyloides* infection in the ferret may lead to useful information regarding the pathogenesis of this important infection. Currently, the ferret can be used as a source for larvae, and be maintained at a relatively low cost.

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CAPSULAR PROTONEPHRIDIA IN MALE *OLIGACANTHORHYNCHUS ATRATA* (ACANTHOCEPHALA)

T. T. Dunagan and R.-M. A. Rashed*
Department of Physiology, Southern Illinois University, Carbondale, Illinois 62901-6503

ABSTRACT: The capsular type protonephridium and associated excretory bladder for male members of a single species in the genus *Oligacanthorhynchus* were examined by scanning electron microscopy. These excretory organs consisted of 2 groups of large, blunt, tightly packed flame bulbs covering a capsule-like chamber located on the anterior end of an expandable bladder. The entire complex was located on the dorsal surface at the junction of the dorsal ligament sac and genital sheath. Each protonephridium consisted of approximately 300–400 flame bulbs with the entire complex measuring 0.3–0.4 mm in length by 0.14–0.25 mm wide. Several electron micrographs are presented that show the bladder in different stages of filling. Both the anterior and posterior termini of the bladder appear to be attached to the underlying surfaces. Evidence is presented which suggests that the Oligacanthorhynchidae should be reorganized taking the nephridial type into consideration as a taxonomic characteristic.

Protonephridia in Acanthocephala are restricted to the family Oligacanthorhynchidae. Schmidt (1972) reviewed this family as well as the class Archiacanthocephala to which it belongs. His keys to the orders of the class included the presence or absence of protonephridia. However, he did not use the type of protonephridia (i.e., dendritic or capsular) to aid in the separation of genera. This organ distinguishes the 8 genera mentioned by Schmidt (1972) into 2 groups: (1) capsular: *Oligacanthorhynchus, Pachysentis, Prosthenorchis*; (2) dendritic: *Nephridiorhynchus, Macracanthorhynchus, Oncicola, Neonicola, Tchadorhynchus.* The dendritic type was discovered first in *Macracanthorhynchus hirudinaceus* by Bojanus (1821), according to Kaiser (1892), and has since been illustrated by Kaiser (1893), Rauther (1930), Meyer (1932), etc. These structures have more recently been studied by Dunagan and Miller (1985, 1986) using scanning and transmission electron microscopy.

The capsular type system was first proposed by Meyer (1931a), according to von Haffner (1942). Meyer in that same year (1931b) described several new species of *Oligacanthorhynchus* (=*Echinopardalis, Nephridiacanthus*) and *Pachysentis* containing this capsule type. He illustrated the protonephridia in most of these species in an abbreviated fashion but included a more detailed drawing (fig. 49) of this organ (minus the bladder) for *Pachysentis procumbens.* Meyer's earlier (1931a) illustration of the protonephridial capsule for *Oligacanthorhynchus taenioides* suggested a much smaller flame bulb than that observed for this system in *P. procumbens.*

The purpose of this paper is to describe a capsular protonephridial organ in *Oligacanthorhynchus atrata* sp. using scanning electron microscope techniques.

MATERIALS AND METHODS

Living material was collected from "street dogs" exterminated in the districts of Torah, El Basateen, and Maasara in Cairo, Egypt, during 2 periods. The first was between 30 August and 31 December 1986. During this period, 3,723 dogs (*Canis familiaris*) were examined, and 1 dog from each of the above districts was infected with *Oligacanthorhynchus atrata.* The second period was between 30 March and 18 April 1987 during which 1,637 dogs were examined and 2 dogs from the district of El Basateen were infected. A total of 44 worms were collected (24 female, 20 male). After washing in tap water, the worms were either fixed in AFA or 2% glutaraldehyde. For purposes of electron microscope studies, worms were postfixed for 2–4 hr in buffered 1% osmium tetroxide at room temperature. Specimens were dehydrated in a graded ethanol series and critical point dried in a Tousimis Samdri model 790 unit with liquid CO_2 as the transitional fluid. After mounting, worms were coated with 40 nm palladium/gold in a Technics Hummer V sputter coater, then examined in a Hitachi S-570 scanning electron microscope at an acceleration voltage of 20 kV. Images were made using Polaroid type 55 film. Measurements were made using the interval scale marker on the S-570 and subsequently enlarged proportionately during printing.

RESULTS

Protonephridia of the capsular type are located on the dorsal surface at the junction of the dorsal ligament sac and the genital sheath in males and the anterior rim of the uterine bell in females. The exact location of each of the 2 protonephrid-

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* Ain Shams University, Faculty of Science, Zoology Department, Cairo, Egypt.

ia relative to each other varies according to the degree of inflation of the bladder. When the bladder is empty or nearly so, they are adjacent and dorsal (Fig. 6) but separate and become dorsolateral (Fig. 1) as the bladder fills. Moreover, during the filling process, the protonephridia may separate from the bladder and extend into the pseudocoel, appearing to be attached only at their anterior terminus (Figs. 1, 4). The shape of the capsule is predominantly oblong (Figs. 1, 2, 4), but other shapes also occur, e.g., obovate (Fig. 6). Measurements of capsules from 6 male worms were: length, 312–400 μm; width, 138–253 μm; thickness, 80–125 μm.

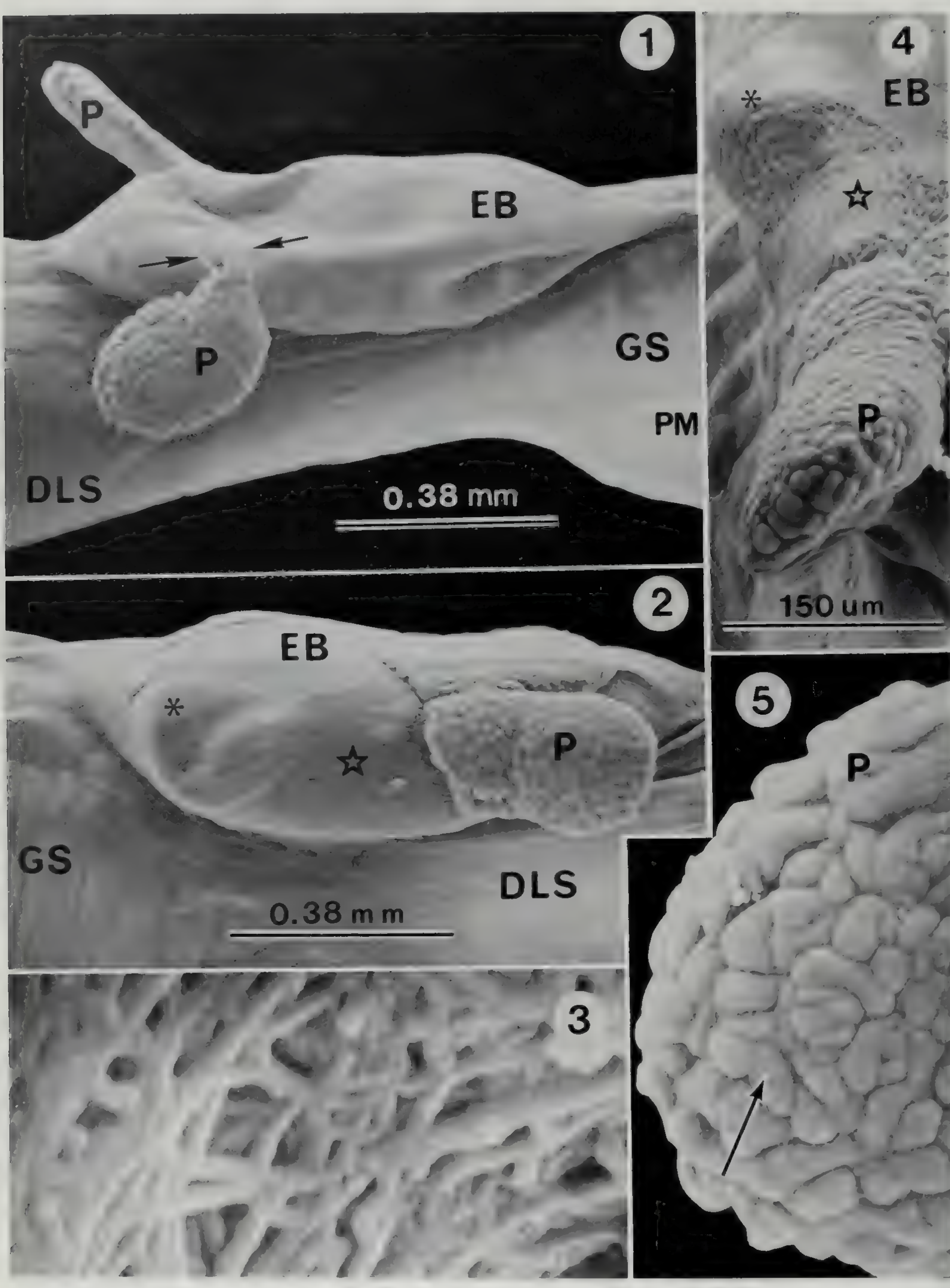
Flame bulbs are located on all capsular surfaces but primarily on those that are not adjacent to the bladder. They are tightly packed (Figs. 4, 5) irregularly shaped "mounds" that are only slightly elevated above the bladder surface. Accurate measurement of the number of bulbs was not possible because of the proximity of the ventral surface to the bladder in the area of capsule attachment. Flame bulb counts of 6 capsules in 4 different male worms varied from 315 to 407. No pattern in the organization of these structures was evident. The external surface of each bulb appears to be composed of an interwoven mat of fibers (Fig. 3). The organization of this mat varies from bulb apex to capsular surface with the tightest weave at the apex and the most open near the capsule (Fig. 3). This pattern creates a number of openings in the outer surface, the size and shape of which vary considerably.

The excretory bladder (Figs. 1, 2, 4, 6) is highly variable in shape and size depending on the degree of inflation (Figs. 6–8 vs. 1, 2, 4). Measurements of 4 male bladders were: length, 0.7–1.3 mm; width, 0.45–0.55 mm; depth, 0.06–0.23 mm. Certain areas on the surface appear to be capable to further inflation, forming knobs (asterisk; Figs. 2, 4) or finger-like extensions. Moreover, the wrinkled appearance (star; Figs. 2, 4) frequently observed also supports this conclusion. A close examination of these wrinkles (Figs. 9, 10) suggests that the wall of the bladder is also composed of a tightly woven mat of fibers that differs from the wall of the flame bulb only in the tightness of the weave. Finally, note that the anterior terminus of the bladder inserts on the dorsal ligament sac and the posterior terminus inserts on the genital sheath (Figs. 1, 2). We believe the bladder is also permanently attached to the underlying genital apparatus between the aforementioned inserts.

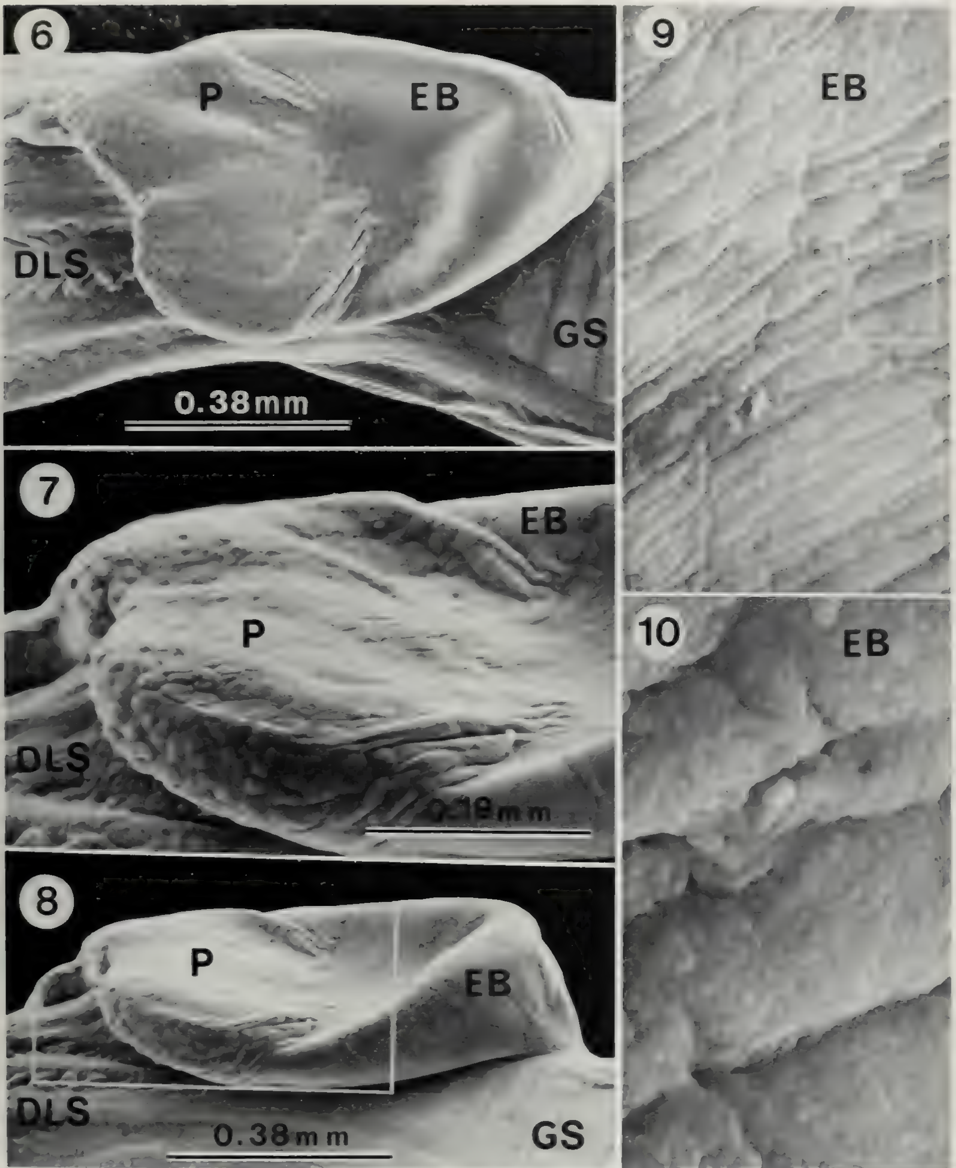
A single specimen depicted capsular processes extending (arrows) for a short distance over the dorsal surface of the bladder (Fig. 1). We were unable to judge from these preparations whether these were "aberrant" flame bulbs or "misdirected" connections with the bladder. They do not appear to enter the bladder.

DISCUSSION

These are the first electron micrographs of a capsular type protonephridial system in an acanthocephalan to be published. These images differ considerably from those depicted by Meyer (1931a) in his description of a new nephridial type in *Oligacanthorhynchus taenioides.* In that same year Meyer (1931b) published a description of several new species that contained a capsule type protonephridial system. Most of his illustrations of this system in these different species are small and unclear. His text is brief, i.e., he stated concerning *Echinopardalis atrata* "mit kapselförmigen Protonephridialorgan" (p. 105) or concerning *Echinopardalis elegans* "mit kapselförmigen Protonephridialorgan ca. 0,25 mm lang" (p. 108). However, his illustration (fig. 49, p. 93) for the protonephridial organ in *Pachysentis procumbens* is more detailed, and his description stated that it was "0,12 mm lang geschlossene Kapsel mit radiären Kölbchen und mit 3 Kernen" (p. 94). More recently, Machado Filho (1950) revised the genus *Prosthenorchis* whose members, as far as we know, all contain a capsule type protonephridial system. He also described several new species belonging to this genus. However, most of his descriptions do not mention or illustrate the presence of a nephridium. The 2 that do, *P. lemuri* and *P. dollfusi,* only illustrate this structure and the drawings (figs. 91, 99) are too small to tell the type. A comparison of these previous observations with this study indicates that this capsule is much larger than those previously described. Moreover, earlier descriptions tend to give only the length of the capsule. Meyer's (1931b) illustration of the nephridium of *Pachysentis procumbens* is the description that most agrees with our observations rather than *Oligacanthorhynchus* (=*Echinopardalis*) *atrata.* Namely, the flame bulbs are large, blunt, closely packed appendages covering the surface of the capsule. This is a departure from Meyer's earlier (1931a) description of *O. taenioides* nephridium which had a capsule design but with a much different size and shape of flame bulbs.



FIGURES 1–5. SEM photographs of protonephridial system in male *Oligacanthorhynchus atrata* showing its capsular design. **1.** Dorsolateral view showing 2 protonephridial capsules along with a slightly deflated excretory bladder. **2.** Lateral view showing knoblike separate expansion of bladder (asterisk) and associated multifolded surface (star). **3.** Surface of flame bulb showing absence of membrane (×20,000). **4.** Portion of specimen in Figure 2 viewed from anterior end. **5.** Enlargement (×400) of protonephridial capsule observed in Figure 1.



FIGURES 6–10. SEM photographs of protonephridial system in male *Oligacanthorhynchus atrata*. **6.** Dorsal view of collapsed bladder. Notice that capsules overlap slightly. **7.** Enlargement of capsular protonephridia of Figure 8. Note how capsule blends with bladder. **8.** Lateral view of Figure 6. **9.** Surface folds in bladder (× 5,000). **10.** Portion of bladder from Figure 9. Note fibrous nature of surface and tightness of the weave (× 20,000). DLS, dorsal ligament sac; ED, excretory bladder; GS, genital sheath; P, capsular protonephridium.

←

Notice the variety of flame bulb (arrow) shapes. DLS, dorsal ligament sac; EB, excretory bladder; GS, genital sheath; P, capsular protonephridium; PM, protrusor muscle.

Taxonomists seem to agree that the Oligacanthorhynchidae comprise a difficult group of worms to separate into genera. We believe an examination of the nephridium would help solve some of this difficulty. A recent example of this problem concerns the placement of *Echinopardalis lamasi* Freitas and Costa, 1964, into *Oncicola* by Schmidt (1977) and the subsequent assignment of this species to *Oligacanthorhynchus* by Amato et al. (1979). None of these authors discussed or illustrated the nephridial type. *Echinopardalis* was erected by Travassos in 1917, but evidence suggests that he never properly recognized the excretory system. However, Meyer (1931b) did recognize this structure and identified it as capsular in this genus. If *E. lamasi* has a capsular protonephridium, its relegation to *Oncicola,* which has a dendritic type protonephridium, appears to be improper. On the other hand, if *E. lamasi* has a dendritic system and was initially improperly placed in *Echinopardalis* by Freitas and Costa (1964), its assignment to *Oncicola* does not violate the nephridial type and Amato et al. placement may be in error. Moreover, Bisseru (1956, p. 44) described *Echinopardalis lerouxi* as "... protonephridial system consists of a branching mass of bulbous structures ...," which we interpret as being dendritic. Schmidt (1972) synonymized *Echinopardalis* with *Oligacanthorhynchus* and transferred *E. lerouxi* to the latter which was appropriate since he moved the genus. However, we believe the original placement was in error, and therefore its current placement should be reconsidered.

One could argue that the nephridial type is of no taxonomic consequence and that species with a capsular or dendritic nephridium should be placed in the same genus. However, we believe different types of nephridia are reflective of greater genetic diversity than such a common placement would suggest. We recommend that the type of nephridium should be given serious consideration as a taxonomic characteristic in the Oligacanthorhynchidae. A comprehensive taxonomic reconsideration of members of this family which would take into account the nephridial types along with other characteristics would be a logical next step.

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RESEARCH NOTES

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Invasion of Murine Dendritic Cells by *Leishmania major* and *L. mexicana mexicana*

R. O. Williams, Imperial College at Silwood Park, Ascot, Berks SL5, 7PY, United Kingdom. Present address: Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, United Kingdom

Species of the genus *Leishmania* are protozoan obligate parasites that reside within cells of the mononuclear phagocyte series. Entry into the host cell is generally believed to be facilitated by the action of phagocytosis (Bray, 1983, Journal of Protozoology **30:** 314–322), though it is not known to what extent the parasite plays an active role. Neither amastigotes nor promastigotes possess any identifiable penetrating organelles, therefore it is surprising to find reports of parasites within nonphagocytic or weakly phagocytic cells. For example, promastigotes of *L. braziliensis* were able to enter human skin fibroblasts *in vitro* (Chang, 1978, American Journal of Tropical Medicine and Hygiene **27:** 1084–1096) and *L. m. mexicana* promastigotes were shown to enter Sticker dog sarcoma cells *in vitro,* despite the addition of cytochalasin B, an inhibitor of phagocytosis (Lewis, 1974, Annals of Tropical Medicine and Parasitology **68:** 327–336). More recently, Ridley and Wells (1986, American Journal of Pathology **123:** 79–85) noticed the presence of amastigotes in what they claimed were dendritic-like cells in lesions of humans infected with *L. m. mexicana* and *L. major*. The purpose of this work was to identify the presence or absence of amastigotes within dendritic cells (DC) from the spleens and lymph nodes of infected mice and to see whether promastigotes would enter DC *in vitro*. DC are currently receiving attention because of their role as antigen-presenting cells. They are considered to be similar to, yet distinct from, macrophages, their chief similarity being their antigen-presenting cell function and their chief difference, their lack of phagocytic ability.

The only infection studied was *L. major* (MHOM/SA/82/RKK; Killick-Kendrick et al., 1985, Transactions of the Royal Society of Tropical Medicine and Hygiene **79:** 252–255) in Balb/c mice. The mice had been infected intradermally in the footpads 3–6 mo before. Single cell suspensions in Hanks' balanced salt solution from spleens or lymph nodes draining the site of infection were layered onto a hypertonic solution of 14.5% sodium metrizamide and centrifuged for 20 min at 600 *g*. The low-density interface cells were then collected, washed 3 times, and cultured for 2 hr in RPMI-1640/10% foetal calf serum (FCS) at 37 C. Nonadherent cells were removed by aspiration and the adherent cells cultured for a further 18 hr. Cells nonadherent after this time, while enriched for DC, contained contaminating monocytes. Finally, Fc receptor (FcR) rosetting was carried out using sheep erythrocytes coated with anti-sheep erythrocyte antibody (GIBCO Ltd., Paisley, Scotland). Identification of DC was on the basis of morphology by interference-phase microscopy and the absence of FcR.

To see if amastigotes could be seen in DC from infected mice, cytocentrifuge preparations were made of the cell suspension containing rosetted and nonrosetted cells. These were washed, fixed in Bouin's fluid, washed again, and stained for 1 hr in 10% Giemsa's stain. After differentiation in acetone and water (1:1), the slides were dehydrated in acetone, cleared in xylene, then euparal essence, mounted in green euparal, and examined microscopically for the presence of parasites in nonrosetted cells.

For *in vitro* work, *L. major* (MHOM/SA/82/RKK) and *L. m. mexicana* (MNYC/BZ/62/M379; Biagi, 1953, Medicina, México **33:** 401–406) were used. Promastigotes of these species were maintained by culture in Schneider's *Drosophila* medium supplemented with 20% FCS. To see whether promastigotes invaded DC *in vitro* a DC-enriched suspension from the spleens of uninfected Balb/c mice were produced as described above. These cells were cultured, prior to FcR rosetting, for 12 hr at 37 C in RPMI-1640/10% FCS with promastigotes at a parasite : cell ratio of 10:1. After FcR rosetting, cytocentrifuge preparations were made that were stained as for amastigotes.

The cell fractionation procedure employed revealed a population of cells that adhered to plastic during the first 2 hr of culture but became nonadherent after 18 hr, lacked FcR, and resembled the DC described by a number of authors, including Steinman and Cohn (1973, Journal of Experimental Medicine **137**: 1142–1162), although the DC morphology was not identifiable in the cytocentrifuge preparations. The DC population constituted less than 1% of the starting population of spleen and lymph node cells.

DC from the lymph nodes of Balb/c mice infected with *L. major* were shown to contain parasites, the percentage of infected cells amounting to 1% of total DC. The number of parasites within each cell was never more than 3 and both intact and disrupted amastigotes were seen. Amastigotes were not seen within spleen DC, though they were found within spleen FcR-positive cells. The presence of parasites within lymph node DC may be related to the fact that there is regular transfer of dendritic-type cells from the skin to the draining lymph nodes (Drexhage et al., 1979, Cell and Tissue Research **202**: 407–430). It may be, therefore, that parasites enter the cells at the site of infection and are transported via the afferent lymphatics to the draining lymph nodes.

After overnight incubation of the DC-enriched suspension with promastigotes of *L. major* and *L. m. mexicana*, parasites were seen within some 5% of the FcR-negative cells. Generally, there were between 1 and 3 parasites per cell, though occasionally more were seen. Parasite membranes, seen under high power, were relatively intact, suggesting that the promastigotes were not killed by the host cell.

It is theoretically possible that finding amastigotes within nonrosetted cells may result from saturation of the FcR of macrophages by antibody or disruption of these receptors by the presence of the parasite. This seems unlikely, since many rosetted cells containing parasites were seen. Another possibility is that a small proportion of DC are phagocytic or that entry of the amastigotes is by endocytosis. It is also possible that the parasites can actively penetrate cells or are capable of inducing phagocytosis in cells normally regarded as nonphagocytic.

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Intestinal Parasites of Small Mammals from Grand Teton National Park

Aelita J. Pinter*, William D. O'Dell, and Raychel A. Watkins, Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana 70148. *To whom correspondence should be addressed

ABSTRACT: A study of the prevalence and identity of *Giardia* spp. in small mammals of Grand Teton National Park was undertaken. All 90 montane voles examined were positive for *Giardia*, as were 4 pocket gophers, 1 water shrew, 4 water voles, and 2 meadow voles. How and why these findings contrast with the findings of others are discussed.

During late summer of 1985, we examined the intestinal contents of 6 montane voles, *Microtus montanus*, and of 1 pocket gopher, *Thomomys talpoides*, that had been trapped as part of a continuing study of microtine populations in Grand Teton National Park (Pinter, 1986, Canadian Journal of Zoology **64**: 1487–1490). Direct examination of the fresh intestinal contents failed to demonstrate any parasites. However, direct light and scanning electron microscope examination of the formalin-fixed duodenal mucosa revealed a heavy infection of *Giardia* spp. in 2 voles and in the pocket gopher. Because of these observations, we initiated a study of the prevalence and identity of *Giardia* spp. in small mammals of Grand Teton National Park.

Study sites were located in Grand Teton National Park at an approximate elevation of 2,057 m. The animals were livetrapped (unbaited Sherman live traps) in late May and again from mid-July to mid-August 1986 and 1987. All of the trapping sites were within a 1,200-m radius. Fresh wet-mount preparations made with scrapings of the mucosa from the first 1–2 cm of the duodenum, from the ileum 1–2 cm above the cecum,

and from the cecum were examined by light microscopy. Remaining portions of the duodenum were fixed in sodium acetate–acetic acid–formalin preservative (SAF, Yang and Scholten, 1977, American Journal of Clinical Pathology **67:** 300–304), while the cecum and contents were preserved in 10% formalin. For light microscopy, impression smears and cecal contents were stained with iron hematoxylin. Prior to staining, the cecal contents were strained through a 20-μm nylon mesh before being concentrated either by centrifugation (which was followed by 2 washes in 10% buffered saline) or by filtration through a 5.0-μm filter (Spaulding et al., 1983, Journal of Clinical Microbiology **18:** 713–715). *Giardia* spp. were identified on the basis of the shape of the median bodies and the length and width of the trophozoites (Filice, 1952, University of California Publications in Zoology **57:** 53–146; Grant and Woo, 1978, Canadian Journal of Zoology **56:** 1348–1359).

Regardless of the host species the median bodies were claw-shaped in all of the *Giardia* trophozoites. The mean length of 28 trophozoites from 4 *M. montanus* hosts was 12.5 μm while the mean width was 7.2 μm. Trophozoites were teardrop-shaped with a flexible pointed posterior. Using the identification scheme of Grant and Woo (1978, loc. cit.), these are *G. microti.* Filice (1952, loc. cit.) recognized this morphological type as belonging to *G. duodenalis.*

All of the 90 montane voles (66 males and 24 females, adults and subadults) were positive for *Giardia.* Living *Giardia* were numerous in wet-mount preparations from the duodenum of 89 animals, including 1 host that had been dead for at least 4 hr. *Giardia* were found not only swimming free but also attached to mucosal cells. No *Giardia* trophozoites (and very rarely cysts) were observed in the wet mount or stained cecal preparations.

Examination of the cecal contents of 39 freshly killed montane voles revealed very heavy infections of trichomonads. Twenty-one montane voles also had infections of *Spironucleus* spp. *Giardia* was found in 4 of 9 pocket gophers, *T. talpoides,* and in 1 of 8 water shrews, *Sorex palustris. Entamoeba* spp. trophozoites and cysts were observed in filtered and stained preparations of the cecal contents of 8 montane voles and of 2 pocket gophers. Two ground squirrels, *Spermophilus armatus,* had heavy trichomonad infections but did not contain any *Giardia.* Four water voles, *Microtus richardsoni,* and 2 meadow voles, *Microtus pennsylvanicus,* were positive for *Giardia* spp. Seven of the 8 water shrews also had infections of *Cochlosoma* spp., a flagellate genus previously described only from birds (Travis, 1938, Journal of Parasitology **24:** 343–351).

Giardia has not been previously reported from *M. montanus.* The high incidence of *Giardia* that we found in *M. montanus* is not unusual among the voles. In an analysis of fecal samples, Wallis et al. (1984, Journal of Wildlife Diseases **20:** 279–283) reported *Giardia* from only 2 of 6 meadow voles, *M. pennsylvanicus.* However, when they examined intestinal scrapings from red backed voles, *Clethrionomys gapperi,* they found *Giardia* in 20 of 21 animals. Grant and Woo (1978, loc. cit.) found nearly a 99% infection rate in *M. pennsylvanicus* when they examined mucosal scrapings. The differences in the incidence of *Giardia* reported in these 2 studies can be accounted for by the method of detection. Like *M. pennsylvanicus, M. montanus* harbor large numbers of trophozoites but shed cysts either only intermittently or in very low numbers. In contrast to the trichomonads or to *Spironucleus, Giardia* trophozoites remain active for several hours after the death of the host so that direct examination of mucosal scrapings or staining of impression smears are better methods for detection than the direct examination of fecal samples.

The reported inverse relationship in densities of *Giardia* to *Hexamita* in the same host (Owen et al., 1979, Gasteroenterology **76:** 757–769) was also observed in 10 of the 21 montane voles with *Spironucleus* infections. In 1 montane vole, *Spironucleus* was the dominant parasite in the duodenum, while *Giardia* dominated in the ileum. In contrast, the locations of *Spironucleus* and *Giardia* were completely reversed in the other 9 animals. The remaining 11 montane voles had mixed infections without any obvious differences in the relative numbers of parasites.

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Possible Association Between *Dirofilaria tenuis* Infections in Humans and Its Prevalence in Raccoons in Florida

Ramiro Isaza and Charles H. Courtney, Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610-0137

ABSTRACT: A study was undertaken to determine the prevalence of *Dirofilaria tenuis* in raccoons from subtropical southern Florida as compared to the northern part of the state. The results indicate that *D. tenuis* is more prevalent in southern Florida and suggest that this might be why humans suffer from more *D. tenuis* infections in southern Florida than in other parts of the state.

The raccoon filariid, *Dirofilaria tenuis,* causes subcutaneous and conjunctival nodules in humans (Gutierrez, 1984, Human Pathology **15:** 514–525). Although infections of humans have been reported throughout the United States, 25 of the 33 (76%) reported cases originated in Florida, and 19 of 22 (86%) infections reported from specified areas of Florida were from southern Florida (Beaver and Orihel, 1965, American Journal of Tropical Medicine and Hygiene **14:** 1010–1029; Jung and Espenan, 1967, American Journal of Tropical Medicine and Hygiene **16:** 172–174; Anderson et al., 1968, Canadian Medical Association Journal **98:** 788; Pacheco and Schofield, 1968, American Journal of Tropical Medicine and Hygiene **17:** 180–182; Rywlin, 1968, Archives of Dermatology **98:** 313; Rywlin et al., 1968, Archives of Dermatology **97:** 425–427; Werth et al., 1971, Texas Medicine **67:** 86–88; Christie, 1977, New England Journal of Medicine **297:** 706–707; Gutierrez and Paul, 1984, American Journal of Surgical Pathology **8:** 463–465). The increased number of human infections in southern Florida has been noted in many publications, most recently Sauerman and Nayer (1985, Journal of Parasitology **71:** 132–134), but no studies have related these observations to geographical differences in prevalence of *D. tenuis* in raccoons in Florida. The purpose of this study was to determine the prevalence of *D. tenuis* in raccoons from subtropical southern Florida as compared to the temperate northern region of the state because it was thought that the increased occurrence of human *D. tenuis* infections in southern Florida may correlate with an increased prevalence of this parasite in raccoons.

Blood samples were collected from 79 raccoons distributed as follows: 35 samples were collected from Alachua County in temperate northern Florida, and 44 samples were collected from 2 sites in subtropical southern Florida—11 from Big Cypress National Preserve in Collier County and 33 from Cape Canaveral in Brevard County. The samples were examined for microfilariae by the modified Knotts technique, and measurements of microfilariae were recorded in microns. Differences in prevalence by geographic site were analyzed by Fisher's Exact Test.

Microfilariae of *D. tenuis* ($n = 40$), with mean measurements of 385 (SD 12.5) long and 7.66 (SD 0.4) wide, were found in 14 raccoons, including 2 of 35 (6%) from Alachua County, 7 of 33 (21%) from Brevard County, and 5 of 11 (45%) from Collier County. The prevalence of *D. tenuis* from Alachua County was significantly different from that of the combined subtropical sites ($P = 0.01$), and indeed was significantly different ($P = 0.005$) or very nearly significantly different ($P = 0.06$), respectively, from Collier and Brevard counties individually. The prevalence of *D. tenuis* was not significantly different ($P = 0.12$) between the 2 counties within subtropical Florida.

Results from the present study indicate that *D. tenuis* is more prevalent in subtropical southern Florida (Collier and Brevard counties) than in temperate northern Florida (Alachua County). Although the effect of other factors, such as increased exposure of humans to absolute numbers of vectors in southern Florida's subtropical climate or an increased association of humans and raccoons in southern Florida, cannot be ruled out, the results of this study suggest that increased reports of *D. tenuis* infection in humans from southern Florida may be related to the increased prevalence of this parasite in raccoons from that region which, in turn, would lead to an increased exposure of humans to infected insect vectors.

Because only a small number of raccoons were available and ancillary data about the raccoon hosts were not available in all instances, other biases that may affect prevalence of *D. tenuis*

such as different host age and sex distributions between collection sites cannot be ruled out. In view of the small sample size, the results, although statistically significant, must be viewed as preliminary until confirmed by other studies.

Cooperation by the Florida Department of Natural Resources in granting trapping permits and the use of state-owned land is greatly appreciated. Published as University of Florida Agricultural Experiment Stations Journal Series No. 7118.

J. Parasit., 74(1), 1988, pp. 190–191

The Effect of Sera from Cats Infected with *Brugia pahangi* and Subsequently Treated with Levamisole on the Infectivity of Third-stage Larvae of *B. pahangi*

D. A. Denham and Rizwana Samad, London School of Hygiene and Tropical Medicine, Keppel St., London, England WC1 E7HT

ABSTRACT: Cats were treated with levamisole and the infective (L3) stage of *Brugia pahangi*. Serum from infected cats was subsequently tested for its ability to infect jirds. Jirds autopsied at 33 days postmortem showed significant levels of parasitemia. This is contrary to reports of a previous study wherein serum from humans infected with *B. malayi* was found to induce cell adherence and death of the L3.

Levamisole is an interesting compound having both anthelmintic and immunomodulatory properties. It is strongly microfilaricidal against *Brugia* spp. in cats (Mak et al., 1974, American Journal of Tropical Medicine and Hygiene **23:** 369–374; Rogers and Denham, 1976, Journal of Helminthology **50:** 21–28) and against both *Brugia malayi* (O'Holohan and Zaman, 1974, Journal of Tropical Medicine and Hygiene **77:** 113–115) and *Wuchereria bancrofti* in man (Narasimham et al., 1978, Southeast Asian Journal of Tropical Medicine and Public Health **9:** 571–575).

Patients suffering from chronic, clinical *B. malayi* filariasis are normally amicrofilaraemic (mf−ve) and Sim et al. (1983, American Journal of Tropical Medicine and Hygiene **32:** 1002–1012) reported that sera from these patients induced the adherence of human buffy coat cells to, and subsequent death of, the infective larvae (L3) of *B. malayi in vitro*, while serum from microfilaraemic (mf+ve) patients induced neither cell adherence nor worm death. However, if mf+ve patients were treated with levamisole, which removed mf from their blood, their serum induced cell adherence and death of the L3.

L3 incubated in the sera from levamisole-treated patients were not infective if inoculated intraperitoneally (i.p.) into jirds (*Meriones unguiculatus*), presumably because the worms had been opsonized and were subsequently destroyed by jird cells. This seemed to be a phenomenon that could be studied profitably in cats infected with *Brugia pahangi*.

The experimental plan was to treat cats with levamisole and determine whether L3 incubated in their serum were infective to jirds. Two cats, infected with 100 L3 of *B. pahangi* each, were selected because they exhibited a stable microfilaraemia. Cat 330 had been infected for 18 mo and cat 333 for 13 mo with mf counts of about $0.8/mm^3$ and $10/mm^3$, respectively. Both cats were treated on 5 consecutive days by the subcutaneous injection of 12.5 mg levamisole/kg. Both cats salivated and were quiescent after each injection but otherwise showed no adverse reactions to treatment. The mf counts of cat 330 fell to about $0.5/mm^3$ but never reached 0, whereas cat 333 became mf−ve for 3 wk and later had only the occasional mf in 100 mm^3 of blood. Serum was collected from both cats 1, 2, 3, 4, and 8 wk post-treatment. One hundred L3 were incubated in 5 0.25-ml samples of each of the 10 test sera. Incubations were for 90 min at 37 C in an atmosphere of 5% carbon dioxide in air, and the L3 were then washed 3 times in

medium 199 by gravity precipitation. A pool of normal cat serum prepared from 10 cats that had never been infected with *B. pahangi* was used as a control. Each of the 55 batches of the serum-treated L3 was inoculated i.p. into individual female jirds which were autopsied 33 days after challenge and the worms recovered and counted. Group means were compared using Student's *t*-test.

A statistical analysis showed that at no time point was there a significant difference ($P < 0.05$) between the recoveries of worms from jirds inoculated with L3 treated with serum from the 2 levamisole-treated cats (i.e., recoveries from all 10 groups were statistically homogeneous). Thus, the results from all 50 jirds given L3 treated with serum from levamisole-injected cats can be considered together. Jirds infected with larvae exposed to normal cat serum yielded a recovery of 46.6 (SD, 22.5) and the mean recovery from all 50 jirds infected with L3 incubated in serum from levamisole-treated cats was 44.8. There was no statistically valid difference between any group. Actual mean recoveries from each time point (10 jirds) were: 1 wk post-treatment (pt) 42.4 (SD, 22.6), 2 wk pt 30.0 (SD, 15.6), 3 wk pt 50.8 (SD, 22.3), 4 wk pt 51.1 (SD, 25.5), and 8 wk pt 49.8 (SD, 22.7).

Clearly the phenomenon described by Sim et al. (1983, loc. cit.) was not evident in our experiment. It is difficult to explain why this should be. Apart from the use of feline rather than human sera, the major difference between the 2 protocols was that we autopsied our jirds 33 days after infection, whereas Sim et al. autopsied their jirds at 60 days. We cannot imagine why this should make any difference, because if the L3 had been opsonized by the feline serum, they would have been attacked immediately after injection into the jirds. It is possible that feline immunoglobulins do not bind to Fc receptors on jird cells, whereas human immunoglobulins do. In retrospect we are even more surprised by our inability to repeat Sim et al.'s results because we have since shown (unpubl.) that cats treated with levamisole are subsequently resistant to challenge with L3 and do not become mf+ve.

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Lack of a Specific Humoral Antibody Response in *Leiostomus xanthurus* (Pisces: Sciaenidae) to Parasitic Copepods and Monogeneans

Dennis A. Thoney and Eugene M. Burreson, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062

Specific antibody production by fish against either parasitic copepods or monogeneans has not been previously demonstrated. However, other data suggest that there are immune responses to their parasites. For example, Shields and Goode (1978, Crustaceana **35:** 301–307) and Shariff et al. (1986, Journal of Fish Diseases **9:** 393–401) suggested that an immunological response may develop in fishes against the copepod *Lernaea cyprinacea.* The picine hosts of the monogeneans *Dactylogyrus vastator* (Paperna, 1964, Bamidgeh **16:** 120–141) and *Gyrodactylus alexanderi* (Lester, 1972, Journal of Parasitology **58:** 717–722) lose their parasites along with a layer of mucus and thus an immunological response may not have been involved. Nigrelli and Breder (1934, Journal of Parasitology **20:** 259–269) and Nigrelli (1937, Zoologia, N.Y. **22:** 185–192) suggested that marine fishes acquire immunity to the monogenean *Epibdella melleni.* Scott and Robinson (1984, Journal of Fish Biology **24:** 581–586) and Scott (1985, Journal of Fish Diseases **8:** 495–503) found that some *Poecilia reticulata* were resistant to *G. bullatarudis* following an initial infestation. Hanson (1972, Ph.D. Dissertation. Oregon State University,

Corvallis, 103 p.) found that 2 embiotocid fishes, *Amphistichus rhodoterus* and *Embiotoca lateralis,* produced weak nonspecific antibodies in mucus and serum to the monogenean *Diclidophora embiotocae.* The presence of specific immunoglobulins was not investigated in any of these studies, hence, such findings are only indirect evidence for a humoral response.

The development of enzyme-linked immunosorbent assay (ELISA), an extremely sensitive technique, has allowed detection of antibodies specific to particular antigens (Voller et al., 1979, Dynatech Europe, Borough Housu Ruede Pres. Guernsey, United Kingdom, 124 p.). Bortz et al. (1984, Developmental and Comparative Immunology **8:** 813–822) have detected a specific humoral immune response in *Salmo gairdneri* against the digenean *Diplostomum spathaceum* using ELISA. The purpose of this study was to determine, by use of a modification of ELISA (Burreson and Frizzell, 1986, Veterinary Immunology and Immunopathology **12:** 395–402), if spot, *Leiostomus xanthurus,* produce specific antibodies to the tissue-dwelling copepod *Lernaeenicus radiatus* or to the gill-inhabiting monogenean *Heteraxinoides xanthophilis.*

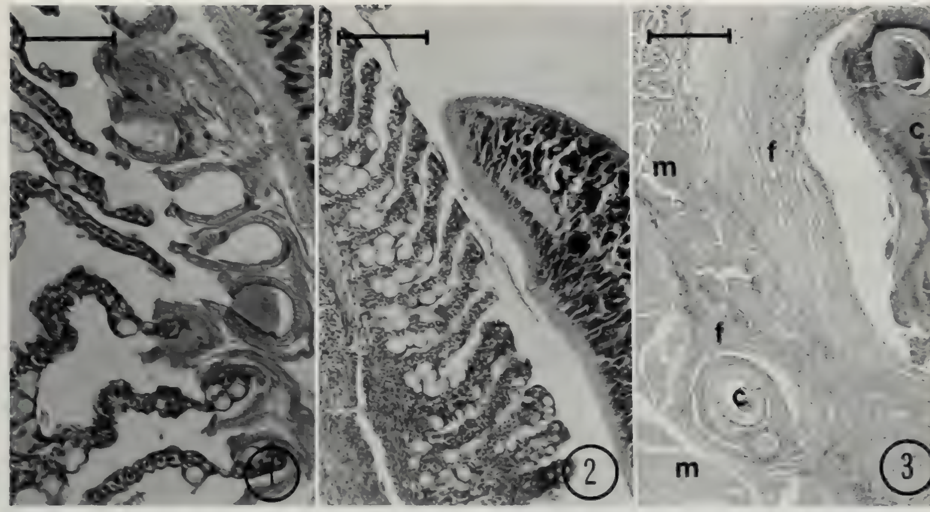
Adult fish used in these assays were collected during summer of 1985 and again during summer of 1986. Postlarvae were collected in April each year and raised in the laboratory in a closed aquarium system. Each year 4 groups of 10 fish each (80 fish total) ($\bar{x}$ fork length = 139 ± 41 mm; range = 91–214 mm) were necropsied. The first group consisted of the spot raised in the laboratory and thus never infested with either of these parasites. This group served as a negative control. The second group consisted of spot collected in the field found to be uninfested with the parasites concerned, but which may have been infested previously. The third and fourth groups consisted of spot collected in the field and harboring *L. radiatus* or *H. xanthophilis,* respectively. Blood was collected by caudal puncture. Serum from each fish was frozen individually at −21 C until analyzed. Each fish was then necropsied to determine intensities of parasites. Tissues of spot infested with *H. xanthophilis* and *L. radiatus* were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin to determine pathological changes associated with these parasites.

Following purification of spot Ig by $(NH_4)_2SO_4$ precipitation, rabbit anti-spot Ig serum, anti-copepod serum, and anti-monogenean serum were prepared. Rabbit anti-spot Ig was prepared by immunizing a rabbit 3 times with spot Ig dissolved in 1.0 ml of PBS and emulsified with an equal volume of Freunds' complete adjuvant and then challenged with spot Ig in PBS (Burreson and Frizzell, 1986, loc. cit.). Positive controls were rabbit anti-copepod serum and rabbit anti-monogenean serum prepared by immunizing rabbits following the above procedure with 1.0 ml PBS containing 2 sonicated copepod heads or 5 sonicated adult monogeneans, respectively. The negative control was preinjection serum from the same rabbits.

Antigen for each assay was prepared by adding 3 µl of supernatant from 2 centrifuged, sonicated copepod heads or 5 monogeneans in 1.0 ml distilled H_2O to a numbered nitrocellulose membrane (5 mm^2). Two membranes, one with each antigen, were prepared for each fish to be assayed. Both antigens were also assayed with positive and negative control serum. Serum from individual fish in each of the 4 groups, diluted 1/100, was assayed twice for specific antibodies against these antigens as described by Burreson and Frizzell (1986, loc. cit.). Basically, the sequence of incubation solutions after addition of the antigen was spot whole serum, rabbit anti-spot Ig, labelled goat anti-rabbit Ig, and color reagent.

In all 4 assays (replicate assays each year) the positive controls, rabbit anti-spot Ig serum, rabbit anti-copepod serum, and rabbit anti-monogenean serum had antibody titers of 1,600 indicating that the rabbit anti-spot Ig was effective in recognizing spot Ig and that the copepod and monogenean antigen concentration was sufficient. Based on comparison with positive and negative controls, antibodies specific to either copepod or monogenean antigen were not detected in any of the spot serum samples suggesting that these parasites were not eliciting a humoral immune response in spot prior to necropsy.

Histological sections of spot gill infested with *H. xanthophilis* showed that there was no cellular response at sites of clamp attachment (Fig. 1) and only slight hyperplasia response in areas where the worm had been feeding (Fig. 2). Evidently, the parasite antigen is not in sufficient quantity and/or quality to elicit a humoral immune response at the infestation intensities observed ($\bar{x}$ intensity = 5.5; range = 2–25). Intensities of *E. mellini* (Nigrelli and Breder, 1934,



FIGURES 1–3. **1.** Longitudinal section of the opisthaptor of *Heteraxinoides xanthophilis* attached to lamellae of gill; scale = 0.15 mm. **2.** Longitudinal section of *H. xanthophilis* showing slight hyperplasia in area of feeding; scale = 0.10 mm. **3.** Cross section of *Lernaeeicus radiatus* embedded in muscle tissue of spot: c—copepod, f—fibrotic tissue, m—host muscle; scale = 0.20 mm.

loc. cit.; Nigrelli, 1937, loc. cit.) were much higher than those of *H. xanthophilis,* and although the intensities of *G. bullatarudis* (Scott and Robinson, 1984, loc. cit.; Scott, 1985, loc. cit.) were similar to those in this study, movements of worms on the skin may affect a much larger surface area in *P. reticulata* than is affected by the stationary *H. xanthophilis* on *L. xanthurus.* In addition, *E. mellini* and *G. bullatarudis* possess hooks and feed on host epidermis both of which may elicit a stronger response. The apparent resistance seen in those hosts may be caused by a nonspecific response to these monogeneans as was demonstrated by Hanson (1972, loc. cit.) in *Diclidophora embiotocae.*

Histological sections of spot muscle tissue infested with mature or dying *Lernaeenicus radiatus* (Fig. 3) show that there is a strong fibrocytic response, effectively encapsulating the head and neck of the parasite in fibrous connective tissue as occurs in *Lernaea cyprinacea* (Tidd and Shields, 1963, Journal of Parasitology **49:** 693–696; Joy and Jones, 1973, Journal of Fish Biology **5:** 21–23; Shields and Goode, 1978, loc. cit.). Although the mouth parts are exposed in live copepods for feeding, encapsulation of the rest of the head and neck may prevent the host's humoral immune system from recognizing the presence of the parasite. It is possible that during the infection process a humoral immune response is elicited, but is inactivated during the process of encapsulation as the parasite is isolated from the surrounding tissue. In this case, memory could be acquired which may make the host more resistant to future infestations, as was seen with *L. cyprinacea* (Shields and Goode, 1978, loc. cit.; Shariff et al., 1986, loc. cit.). Future studies measuring specific antibodies during the early copepod infestation period are needed.

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Phosphoglycerides and Derivatives in *Taenia crassiceps* Examined by ^{31}P NMR Spectroscopy

Stewart N. Thompson*, Edward G. Platzer, and Robert W.-K. Lee, Nuclear Magnetic Resonance Analytical Laboratory, University of California, Riverside, California 92521. *Address for correspondence: Division of Biological Control, University of California, Riverside, California 92521

ABSTRACT: Examination of the larval stage of the tapeworm, *Taenia crassiceps*, by ^{31}P NMR spectroscopy revealed the presence of a major phosphoglyceride component. However, using saturation transfer, no exchange between glycerophosphorylcholine and phosphoglyceride or any other NMR-detectable phosphorus metabolites was detected.

Previous NMR investigations have demonstrated glycerophosphorylcholine to be a major phosphorus component of helminth parasites (Tielens et al., 1982, Molecular and Biochemical Parasitology **6:** 175–180; Rohrer et al., 1986, Archives of Biochemistry and Biophysics **248:** 200–209; Thompson et al., 1987, Molecular and Biochemical Parasitology **22:** 45–54). Mathews et al. (1985, Biochimica et Biophysica Acta **845:** 178–188) outlined the possible metabolic functions of this metabolite including its potential role as a novel precursor or a breakdown product in phospholipid metabolism. Free phosphoglycerides, however, are minor components or were undetected in the NMR spectra of helminths examined thus far. Here we report a major phosphoglyceride component in the ^{31}P NMR spectrum of the larval stage of the tapeworm, *Taenia crassiceps.* Using saturation transfer we were, however, unable to detect any exchange between glycerophosphorylcholine and phosphoglyceride or other NMR-detectable phosphorus metabolites.

Taenia crassiceps, ORF strain, was maintained in the laboratory in white mice and fresh material for analysis was obtained by irrigation of the peritoneal cavity with physiological saline. Cysticerci (2–3 ml) with a dry wt of 75–125 mg were placed in a 12-mm NMR tube containing 5 ml of Kreb's ringer saline (less phosphate) containing 50 mM HEPES, pH 7. ^{31}P NMR spectra were generated at 121 MHz under nonsaturating conditions in a wide-bore Nicolet 300 super-conducting spectrometer as described previously (Thompson et al., 1987, loc. cit.). Tissue viability was examined by monitoring spectral changes and nucleotide triphosphate (NTP) level over time. The cysticerci were not perfused with the buffer and, therefore, experiments were conducted at room temperature (23–25 C) rather than at 37 C. Previous study with *Hymenolepis diminuta* demonstrated that viability was seriously compromised at higher temperature under nonperfused conditions.

The ^{31}P NMR spectrum of *T. crassiceps* was composed of 11 peaks (Fig. 1A). Chemical shifts were established relative to a phosphoric acid standard. Assignment of most components was made on the basis of previous studies (Mathews et al., 1985, loc. cit.; Thompson et al., 1987, loc. cit.). The peaks in the phosphodiester region were presumed to contain phospholipids and worms were extracted with 10% cholic acid as described by London and Feigenson (1979, Journal of Lipid Research **20:** 408–412). Assignments for the spectral components of detergent extracts were made on the basis of chemical shifts as reported by Henderson et al. (1974, Biochemistry **13:** 623–628), and by spiking with pure compounds (Fig. 1B).

The phospholipid composition of extracts was verified by thin-layer chromatography. Phospholipids were extracted from detergent extracts with $CHCl_3$:MeOH (Bligh and Dyer, 1959, Canadian Journal of Biochemistry and Physiology **37:** 911–917) and analyzed by two-dimensional chromatography (Thompson, 1987, Comparative Biochemistry and Physiology **87B:** 357–360) on 250-μm layers of silica gel G (Fig. 2). Individual phospholipids were identified by Rf value, co-chromatography with pure standards, and by use of selective spray reagents including molybdenum blue (phosphorus), Dragendorf's reagent (choline), benzidine–hypochlorite (sphingolipid), and ninhydrin (amino groups). Diphosphatidyl glycerol was not detected on thin layers, possibly because of its position in or close to the solvent front.

Saturation transfer was carried out with a General Electric GN500 spectrometer at 202 MHz. Diphosphatidyl glycerol (peak 4) was selectively

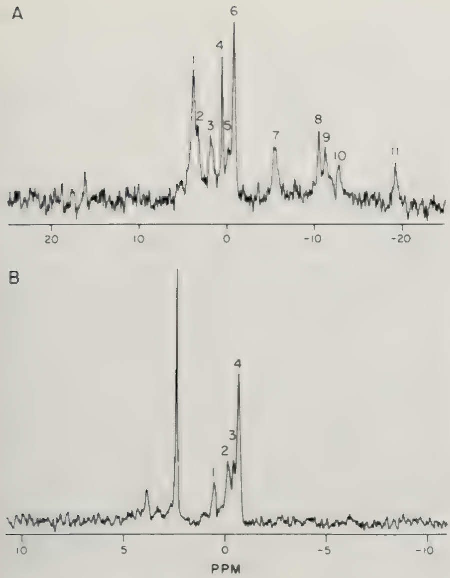

FIGURE 1. ^{31}P NMR spectrum of *T. crassiceps*. **A.** *In vivo* spectrum. Peak assignments are as follows: (1) sugar phosphates, (2) phosphorylcholine, (3) inorganic phosphate, (4) diphosphatidyl glycerol, (5) glycerophosphorylcholine, (6) phosphatidylcholine + lysophosphatidyl choline, (7) β NDP + γ NTP, (8) α NDP + α NTP, (9) NAD + the phosphorus resonance of glucose phosphate from UDP-glucose, (10) phosphorus resonance of uridine phosphate from UDP-glucose, and (11) β NTP. Phosphatidyl ethanolamine and sphingomyelin occur within the regions of peaks 5 and 6 but firm assignment in the *in vivo* spectrum cannot be made at this time. **B.** Spectrum of 5% cholic acid extract. Peak assignments are as follows: (1) diphosphatidyl glycerol, (2) glycerophosphorylcholine, phosphatidyl ethanolamine, and sphingomyelin, (3) lysophosphatidyl choline, and (4) phosphatidyl choline. The large downfield peak is inorganic phosphate. Spectra represent 2,400 data acquisitions.

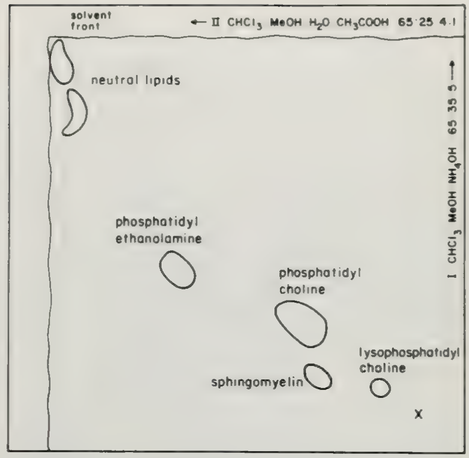

FIGURE 2. Tracing of thin-layer chromatogram of the phospholipids extracted in $CHCl_3$:MeOH from detergent extracts of *T. crassiceps*. Phospholipids were detected with molybdenum blue and neutral lipids by charring after spraying with 50% H_2SO_4.

saturated as shown in Figure 3 by application of a series of low-power 50-msec alternately inverted pulses for 2 sec. Under these conditions the excited nuclei are largely saturated and therefore undetected. Data were accumulated for 16 hr. Examination of the spectrum after that time demonstrated a significant increase in the relative level of inorganic phosphate as well as an upfield shift of approximately 0.78 ppm (Fig. 3 inset). Based on previous titration of helminth extracts, this represented a pH decrease from approximately 7.1 to 6.6. Nevertheless, the tissue was viable as indicated by the maintenance of the nucleotide pool (Fig. 3 inset). Exchange would have been evidenced by negative peaks in the difference spectrum (Fig. 3) due to loss of signal from phosphorus components exchanging with saturated diphosphatidyl glycerol nuclei. Similar negative results were obtained after selectively saturating peak 5 containing glycerophosphorylcholine. The above investigation does not demonstrate conclusively that phosphoglycerides and glycerophosphorylcholine do not exchange but rather that any exchange is occurring very slowly. NMR can only detect exchange if the rate is faster and of the same order of magnitude as the inverse of the spin-lattice relaxation time (T_1). Although T_1 was not determined in the present study, Mathews et al. (1985, loc. cit.) estimated the T_1 for glycerophosphorylcholine in *Fasciola hepatica* to be approximately 10 sec. Further investigation of glycerophosphorylcholine metabolism by NMR may require the use of ^{13}C-labeled phosphoglyceride derivatives. The complex multistep nature of phospholipid synthesis and degradation may make further metabolic studies by ^{31}P NMR unsuitable.

We thank Dr. W. K. Kroeze of Macdonald College for providing the *T. crassiceps* inoculum and Dr. Justin Roberts for the use of the General Electric GN500, which was purchased in part by

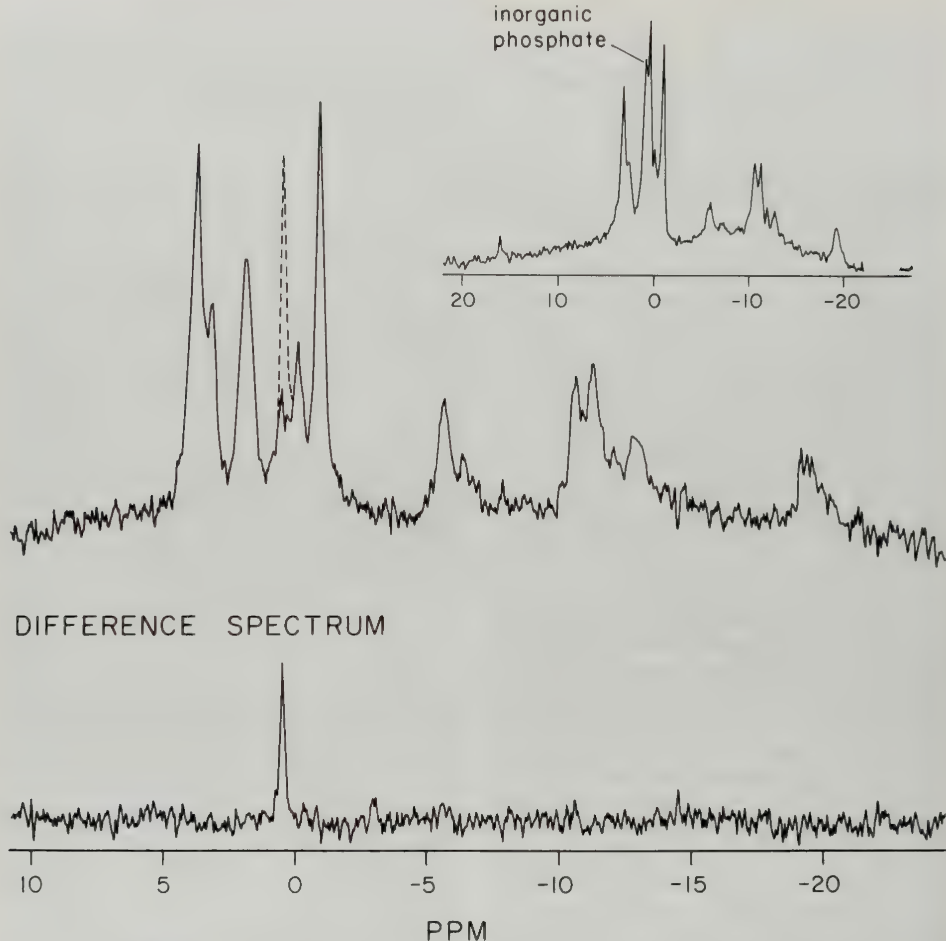

FIGURE 3. ^{31}P NMR spectrum of *T. crassiceps* during saturation transfer experiment. Dotted line shows spectrum before saturation and solid line following selective saturation of diphosphatidyl glycerol. Difference spectrum was produced by computer subtraction of the saturated from the normal spectrum. Spectra represent several thousand data acquisitions. Inset: Spectrum after 16 hr. Spectrum represents approximately 200 data acquisitions.

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LETTERS TO THE EDITORS . . .

Critical Comments on a Recent Letter to the Editors Regarding the Use of Frozen Carcasses in Parasite Surveys

We take exception to the Letter to the Editors by Shoop et al. (1987, Journal of Parasitology **73**: 109). While these individuals are to be commended for their concern regarding the collection and reporting of data from parasite surveys and we agree with some of their concerns, we disagree with their assessment of the cause of the problem. We are concerned also that an uncritical acceptance of their viewpoint would restrict the use of a valuable technique.

Each of us has used frozen viscera for parasitological surveys for many years. We are well aware of the freezing artifact produced by ice crystal formation. Although formation of ice crystals after relatively slow freezing of specimens may be disruptive to the architecture at the cellular level (Lillie, 1965, Histologic technique and practical histochemistry, McGraw-Hill Book Company, New York, pp. 32–34), we have not experienced such changes at either the organ or organismic level. It is apparent that these changes are not sufficient to lyse, rupture, or otherwise destroy even the smallest cestodes, digeneans, or monogeneans. Examples with which some of us have had extensive experience include ancyrocephalid Monogenea of fishes, microhymenolepid Eucestoda of waterfowl, and microphallid Digenea of shore birds. These helminths are easily ruptured by routine manipulation or coverslip pressure, yet examination of thousands of specimens from frozen hosts, as well as fresh and formalin-fixed material, indicates little evidence of discernible structural damage and certainly not enough to preclude identification. For example, in studies on the Ancyrocephalidae of North America (Mizelle, 1936, American Midland Naturalist **17**: 785–806; 1938, American Midland Naturalist **19**: 465–470) there was extensive use of frozen material in taxonomic descriptions. Examination of type material from these studies presents little difficulty in species determination and the degree of "damage" is comparable to that produced by any of the other fixation techniques; only examination of living specimens provides more complete morphological data, but these are not usually available for study.

We concede that there may be some loss of affinity for stains in acanthocephalans, cestodes, monogeneans, and digeneans, some loss of scolex hooks in cestodes, and some loss of surface spination in digeneans, but there is no evidence for the actual loss of these helminths or of the severe degeneration of specimens ("unrecognizable mush") that form the basis for the concern expressed by Shoop et al. Rather, what these authors describe are postmortem autolytic changes in helminths collected from improperly handled host specimens. Such changes are encountered frequently in helminths from vertebrates, especially well-insulated homeotherms, frozen slowly by placing the whole carcass in the freezer, frozen viscera and carcasses subject to repeated thawing and refreezing, or by leaving the whole carcass in subfreezing ambient temperatures for varying periods of time prior to evisceration and freezing. Also, such changes occur frequently in gastrointestinal helminths from "freshly necropsied" carcasses of animals with high metabolic rates (birds, insectivores, chiropterans), large bodies (herbivores), well-developed insulation (waterfowl, furbearers), or rapid decomposition rates (most fish) examined within a few hours after death, especially in warm and humid climates. In these species, quickly eviscerated and promptly frozen viscera and carcasses are preferable to poorly attended "freshly" necropsied carcasses examined several hours after death.

A recently developed technique is the use of an ethyl alcohol–dry ice mixture (see Bush and Holmes, 1986, Canadian Journal of Zoology **64**: 142–152) to "quick-freeze" viscera. Subsequently, viscera are kept in a freezer until necropsy. Helminth parasites, even the very small platyhelminths, are preserved *in situ* and in their exact location and natural postures at the instant of death. This technique is invaluable for ecological studies on populations and communities of helminth parasites. These specimens are in excellent condition, and are easily identified unfixed with phase-contrast microscopy or stained for subsequent identification. Even following prolonged periods of freezing, with or without dehydration due to "freezer burn," specimens can be rehydrated in water and subsequently identified. Soaking in a trisodium phosphate solution will enhance the subsequent staining of dehydrated specimens.

We believe that freezing of viscera, if properly applied, is an invaluable technique for obtaining and storing vertebrate specimens for parasite surveys. Logistical constraints related to time that can be spent in the field, distance from laboratory facilities, time required for adequate necropsies, size of the animal, etc., are factors that necessitate some form of intermediate storage of vertebrate specimens prior to their necropsy. Freezing or preservation in fixative are the only viable alternatives for the collection of such material. In our experience, working with fixed viscera is unpleasant, somewhat hazardous, and usually results in contracted specimens with adherent mucus that is difficult to remove. Additionally, small parasites such as recently recruited digeneans and cestodes that are in the lumen of the intestine and tissue-dwelling nematodes are difficult to recover and in cases of low parasite abundance, parasite occurrence may be, and abundance will be, underrepresented. Frozen material has none of these disadvantages.

We believe that even inadequately handled frozen material may be useful. Frozen specimens of rare or hard to obtain vertebrates may be available in no other condition, but these may still provide valuable data on parasites. We agree with Shoop et al. that authors using such material should be aware of its limitations, and they should warn the reader of its quality. While these authors state that "It is truly a disservice to the parasitological community when a paper is published purporting to have looked at all parasites of a particular

host species when in fact the methodology only allowed adequate recovery of one or two parasitic groups (sic)," we contend that it is a greater disservice to the parasitological community to condemn unequivocally a method that has contributed so much to the knowledge of helminth faunas worldwide. This controversy could be resolved experimentally by comparing previously known numbers of helminths recovered from frozen versus freshly necropsied viscera. Because both methods are useful and therefore likely to be employed for some time, quantitative estimates of the relative error would be enlightening. Finally, we urge the Editors of parasitological journals to offer concurrent rebuttal and critical comments on Letters to the Editors such as the one by Shoop et al. prior to publication because (in these authors' own words) "once these . . . [letters] are published they are difficult to recognize for what they are and disinformation such as this is misleading to colleagues."

Danny B. Pence, Department of Pathology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430; **John M. Aho,** Savannah River Ecology Laboratory, University of Georgia, Drawer E, Aiken, South Carolina 29801; **Albert O. Bush,** Department of Zoology, Brandon University, Brandon, Manitoba, Canada R7A 6A9; **Albert G. Canaris,** Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968; **Joseph A. Conti,** Oglethorpe County Middle School, Lexington, Georgia 30648; **William R. Davidson,** Southeastern Cooperative Wildlife Disease Study, Department of Parasitology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602; **Terry A. Dick,** Department of Zoology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2; **Gerald W. Esch, Timothy Goater, and Wynn Fitzpatrick,** Department of Biology, Wake Forest University, Winston-Salem, North Carolina 27109; **Donald J. Forrester,** Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610; **John C. Holmes and William M. Samuel,** Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9; **John M. Kinsella,** 2108 Hilda Avenue, Missoula, Montana 59801; **Janice Moore,** Department of Zoology, Colorado State University, Fort Collins, Colorado 91330; **Robert L. Rausch,** Division of Animal Medicine, School of Medicine, University of Washington, Seattle, Washington 98195; **William Threlfall,** Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9; **and Terry A. Wheeler,** Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

J. Parasit., 74(1), 1988, p. 198

Rescue the Word "Pathology" from the Pits!

This is a plea for assistance in rescuing the word "pathology" from the pits of doom wherein resides an overpopulation of misused words with the suffix -ology. Just as it would be incorrect to state, "We didn't see any parasitology" (i.e., nematodes), likewise it is totally improper to write, ". . . the parasites are prevented from invading epithelial cells to cause much pathology . . ." (Journal of Parasitology **73:** 665, 1987, the last sentence).

The word "pathology" does not equal words such as "lesion," "change"; it is a "branch of medicine which treats of the essential nature of disease" (Dorland's Medical Dictionary). I would appreciate your assistance in this rescue effort.

Bruce C. Anderson, Caldwell Veterinary Teaching Center, University of Idaho, Caldwell, Idaho 83605.

J. Parasit., 74(1), 1988, p. 199

ANNOUNCEMENT . . .

11th International Convocation of Immunology
Buffalo, New York, 12–16 June 1988
Immunology and Immunopathology of the Alimentary Canal

The Ernest Witebsky Center for Immunology will present this symposium in its regular biennial series at the Hyatt Regency Buffalo Hotel. Closed plenary sessions will focus on the topics: Basic Immunologic Considerations, Immunologically Responsive Tissue Cells; Immunopathologic Conditions (dental caries; periodontal disease; inflammatory bowel disease; celiac disease; gastrointestinal infections and infestations); Immune Response in Oral and Gastrointestinal Neoplasms; Nutritional Effects on the Immune Response; and Development of Vaccines. Open poster sessions for free contributions on the theme will be offered.

For further information contact: Dr. James F. Mohn, Director, The Ernest Witebsky Center for Immunology, 240 Sherman Hall, State University of New York at Buffalo, Buffalo, New York 14214 (Telephone: 716-831-2848).

J. Parasit., 74(1), 1988, p. 199

ANNOUNCEMENT . . .

The British Society for Parasitology and the Linnean Society

There will be a joint 1-day scientific meeting entitled *Research Developments in the Study of Parasitic Infections* on Thursday, 15 December 1988, at the Linnean Society Rooms, Burlington House, Piccadilly, London W1V OLQ, organized by Professor R. M. Anderson, FRS, Dr. Christine A. Facer, and Dr. D. Rollinson.

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HE OURNAL OF PARASITOLOGY

Founde Ward

CURRENT ISSUE

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AME ETY OF PARASITOLOGISTS

THE JOURNAL OF PARASITOLOGY

The Journal of Parasitology, published bimonthly, is both the official organ of the American Society of Parasitologists and a medium for publication of research primarily on animal parasitology.

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Dr. David F. Mettrick and Dr. Sherwin S. Desser
Editors, The Journal of Parasitology
Department of Zoology
University of Toronto
Toronto, Ontario
Canada M5S 1A1

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THE *IN VITRO* AND *IN VIVO* EFFECTS OF METRONIDAZOLE AND CHLOROQUINE ON *TRYPANOSOMA BRUCEI BRUCEI*

Ijeoma N. Otigbuo and Patrick T. K. Woo*†

Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, P.M.B. 12003, Lagos, Nigeria

ABSTRACT: Bloodstream forms of *Trypanosoma brucei brucei* were grown over baby hamster kidney cells in minimum essential medium with various concentrations of metronidazole (Flagyl) and chloroquine. Both drugs inhibited the multiplication of the parasite *in vitro*. The least effective concentrations for metronidazole and chloroquine were 0.003 mg/ml and 0.0024 mg/ml, respectively. Groups of 12-day-old female CDI mice were treated with 1 of the 2 drugs at 24, 48, and 72 hr after *T. brucei* infection. The drugs administered stat or daily reduced the number of parasites in the mice but did not effect a cure; they prolonged the survival period of the animals. However, metronidazole (0.1 mg/kg body weight) and chloroquine (0.08 mg/kg body weight) combined and given daily for 4 consecutive days cleared the infection. No trypanosomes were detected in the blood of these mice 3 mo after treatment. The dosages for both the *in vitro* (metronidazole 0.003 mg/ml; chloroquine 0.0024 mg/ml) and *in vivo* (metronidazole 0.1 mg/kg body weight; chloroquine 0.08 mg/kg body weight) were well below those prescribed for humans.

The existing drugs that are used against African trypanosomiasis are highly toxic, have serious side effects, and elicit drug resistance in the parasites (Evans, 1981; Meshnick, 1984). Also some of the drugs sometimes fail to cure the disease (Williamson, 1970), and they are generally not readily available (Abaru et al., 1984).

Our interest in chloroquine as a possible trypanocide stems from the successful *in vivo* and *in vitro* trials of this drug and others as possible inhibitors of histamine metabolism (Duch et al., 1984). Nathan et al. (1979) successfully cured *T. brucei* infection with Imidocarb, one of the compounds that inhibited histamine-N-methyl transferase. Because chloroquine also inhibited this enzyme, it is possible that it may also have trypanocidal activity.

The nitroimidazoles, of which metronidazole is a member, are highly effective against protozoa (Cosar and Julou, 1959), and in combination with other drugs, have been shown to be less toxic (Malanga et al., 1981), and curative (Jennings et al., 1983, 1984; Raseroka and Ormerod, 1985). Also, metronidazole has been shown to be effective against *Trypanosoma cruzi* infection (Raether and Seidenath, 1983).

The aims of the present study were to determine (1) the *in vivo* and *in vitro* effects of 2 readily available and inexpensive drugs (metronidazole and chloroquine; both have product licenses in Africa) on *Trypanosoma brucei brucei,* and (2) if the 2 drugs have additive and/or synergistic effects and their potential as trypanocides.

MATERIALS AND METHODS

Trypanosome

A monomorphic strain of *T. b. brucei* (Shinyanga III) was used. The strain was initially isolated in 1946 in Uganda and was maintained by cryopreservation and serial passages in mice, at the University of Guelph, Guelph, Ontario, Canada, since 1967.

Trypanosomes for culture were isolated from mouse blood according to Hirumi et al. (1977). Citrated saline was used as an anticoagulant. The number of trypanosomes in the inoculum was determined using a Coulter counter with aperture set at 70 μm; all counting fluid was filtered (using Millipore filters, 0.22 μm) prior to being used.

Animals

Twelve-day-old female CDI (Charles River Incorporated, Ontario, Canada) mice were used throughout the study.

Culture system

Eagle minimum essential medium (MEM) was supplemented with fetal calf serum (FCS) (Grand Island Biological Company, Industrial Avenue, Burlington, Ontario, Canada). Baby hamster kidney (BHK) cells were used as feeder layer cells. They were obtained from Dr. V. L. Chan (Department of Microbiology, University of Toronto, Toronto, Ontario, Canada) and maintained in plastic Nunc 25-cm flasks with 5 ml of medium. Subcultures were carried out after trypsinization and resuspension of the feeder layer cells. The pH in the medium was adjusted to 7.4, and the medium contained various concentrations of metronidazole and/

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* Department of Zoology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

† Author to whom reprint requests should be addressed.

or chloroquine (Table I). The amount of drug used was initially 1/100,000 the human dosage (chloroquine 200 mg; metronidazole 500 mg). If no effects were detected, the amount was increased.

Washed trypanosome suspension (1 ml) containing 10^3 parasites was inoculated into each culture flash (Hirumi et al., 1977), which contained 24-hr-old subcultured BHK cells. All cultures were maintained at 37 C (95% relative humidity with 5% CO_2 and 95% air). The cultures were examined daily under an inverted microscope. Five-day-old trypanosome cultures were inoculated intraperitoneally into 12-day-old female CDI mice. All experiments were conducted in duplicate and the study was repeated. Smears of flagellates were made from cultures after centrifugation, and from blood of inoculated mice. The smears were fixed in absolute alcohol and then in 10% buffered formalin for 10 min. They were then stained for 40 min in Gurr's improved R66 Giemsa. The smears were examined under an oil immersion objective (×100).

Drugs

Chloroquine and metronidazole were obtained from Dr. Keystone, Tropical Diseases Research Center, Toronto General Hospital, Toronto, Ontario, Canada.

Chloroquine hydrochloride injection—a sterile solution of chloroquine in water for injections was prepared; each ml contained 50 mg of chloroquine base, which was equivalent to 40.5 mg chloroquine. Metronidazole was in a sterile solution (50 mg per 10 ml) suitable for intravenous injection.

Drug treatment of infected mice (single dose)

Ninety-six mice were each infected with 10^3 parasites and groups of 6 treated with either chloroquine or metronidazole or both drugs (Tables II, III). The drugs were inoculated intraperitoneally at 24, 48, and 72 hr after infection. The peripheral parasitemia (in tail blood) was determined at regular intervals after treatment. The drug dosages used in the *in vivo* study were derived from the least effective dose (concentration of drug that does not kill the parasite, permits no increase in number, and prevents those that are present from being infective) determined from the *in vitro* study.

The *in vitro* least effective dose for chloroquine was 0.0024 mg/ml (Table I); therefore the dose for a 30-g mouse (about 1 ml of blood) was 0.0024 mg chloroquine or 0.08 mg/kg body weight. For metronidazole, the least effective *in vitro* dose was 0.003 mg/ml; therefore the dose for a 30-g mouse was 0.003 mg or 0.1 mg/kg body weight.

Multiple doses

Infected mice (n = 42) were divided into groups of 6 animals/drug or drug combination (Table IV). They were treated at the following intervals:

1) 24 and 48 hr postinfection (Group 1);
2) 24, 48, and 72 hr postinfection (Group 2);
3) 24, 48, 72, and 96 hr postinfection (Group 3);
4) 24 and 72 hr postinfection (Group 4);
5) 24 hr with both drugs (metronidazole) (Group 5) and (chloroquine) double the dosage (Group 6);
6) no treatment (Group 7: controls).

TABLE I. *Least effective dose of drugs on* Trypanosoma brucei *cultures (starting inoculum 10^3).*

Drug	Drug level (mg/ml)	Days after exposure to drug with parasite counts								
		1	2	3	5	7	9	11	13	14
Chloroquine	0.04	—	—	—	—	—	—	—	—	—
	0.02	10^3*	—	—	—	—	—	—	—	—
	0.01	2×10^3	—	—	—	—	—	—	—	—
	0.005	10^4	—	—	—	—	—	—	—	—
	0.0024	2×10^4	2×10^4	2×10^4	10^4	2×10^3	2×10^3	2×10^3	2×10^3	2×10^3
	0.0012	10^5	10^7	10^9	10^9	10^{10}	10^{11}	10^{11}	10^{12}	10^{12}
Metronidazole (Flagyl)	0.1	—	—	—	—	—	—	—	—	—
	0.05	10^3	—	—	—	—	—	—	—	—
	0.025	2×10^3	—	—	—	—	—	—	—	—
	0.012	10^4	—	—	—	—	—	—	—	—
	0.006	2×10^4	—	—	—	—	—	—	—	—
	0.003	10^5	10^5	10^4	10^4	10^4	10^4	10^4	10^3	10^3
	0.0015	10^4	10^6	10^7	10^8	10^8	10^9	10^9	10^9	10^9
Metronidazole + chloroquine	0.006 + 0.005	10^2	—	—	—	—	—	—	—	—
Metronidazole + chloroquine	0.003 + 0.002	2×10^3	2×10^2	2×10^2	2×10^2	2×10^2	2×10^2	2×10^2	2×10^2	2×10^2
Metronidazole + chloroquine	0.001 + 0.0015	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	—	—
Controls (no drugs)		10^5	10^6	10^7	10^8	10^9				

* Number of parasites/ml of culture medium.

TABLE II. In vivo *treatment of infected mice with a single dose of drugs.*

Treatment	Age of infection (hr)	Number* of parasites before treatment	Number of parasites after treatment (hr) 24	48	72	96	120	144	168	Time of death after treatment (days)
Metronidazole	24	10^4	10^3	10^5	10^7	10^8	10^9	—	—	4
(0.1 mg/kg body weight)	48	5×10^4	10^8	10^8	10^9	—	—	—	—	3
	72	2×10^7	10^8	10^9	—	—	—	—	—	1
Chloroquine	24	10^4	2×10^2	10^3	2×10^5	2×10^7	2×10^8	2×10^9	—	6
(0.08 mg/kg body weight)	48	5×10^4	2×10^5	2×10^7	2×10^8	2×10^9	—	—	—	4
	72	8×10^6	2×10^8	10^9	2×10^9	—	—	—	—	3
Metronidazole + chloroquine	24	10^4	10^2	10^3	10^3	10^5	3×10^7	3×10^8	3×10^9	7
(0.1 mg/kg + 0.08 mg/kg)	48	5×10^4	10^3	3×10^4	3×10^6	3×10^8	3×10^8	3×10^9	—	6
	72	3×10^8	10^6	10^8	10^9	—	—	—	—	3
Control	24	2×10^4	2×10^5	2×10^8	2×10^9	—	—	—	—	
	48	2×10^5	2×10^7	2×10^9	—	—	—	—	—	
	72	2×10^8	—	—	—	—	—	—	—	

* Per ml of blood.

TABLE III. In vivo *treatment of infected mice with multiple doses of drugs.*

Drug	Groups	Treatment period	Number* of parasites after treatment (hr) 24	48	72	96	120	144	168	192	Time of death after treatment (days)
Chloroquine	1	24, 48	10^4	10^3	10^2	8×10^5	10^7	10^9	—	—	5
(0.08 mg/kg body weight)	2	24, 48, 72	10^4	10^3	8×10^3	10^2	10^2	4×10^5	8×10^8	—	6
	3	24, 48, 72, 96	2×10^4	2×10^3	10^3	10^2	50	10^3	10^5	4×10^8	7
	4	24, 72	10^4	10^3	10^4	10^3	10^5	8×10^8	—	—	5
	5	24 only	10^4	10^3	10^4	10^6	10^9	—	—	—	4
	6	24 only, double dosage	10^4	10^2	10^3	7×10^5	10^8	3×10^9	—	—	5
Metronidazole	1	24, 48	10^4	3×10^3	10^2	10^4	10^7	3×10^9	—		5
(0.1 mg/kg body weight)	2	24, 48, 72	10^4	5×10^3	10^3	10^2	10^3	10^7	10^9	—	6
	3	24, 48, 72, 96	3×10^4	2×10^3	5×10^2	10^2	40	10^4	10^7	2×10^9	7
	4	24, 72	2×10^4	10^3	10^3	90	10^3	10^7	10^9	—	6
	5	24 only	10^4	10^3	10^5	10^8	8×10^8	—	—	—	4
	6	24 only, double dosage	10^4	10^2	10^4	10^7	3×10^9	—	—	—	4

* Per ml of blood

TABLE IV. In vivo *treatment at 24 hr postinfection; multiple doses of a combination of metronidazole (0.1 mg/kg body weight) and chloroquine (0.08 mg/kg body weight).*

Group	Treatment period (hr after infection)	Number* of parasites after treatment (hr)								Time of death after treatment (days)
		24	48	72	96	120	144	168	192	
1	24, 48	10^3	10^2	10^2	2×10^3	4×10^5	10^6	3×10^8	9×10^8	7
2	24, 48, 72	2×10^3	8×10^2	10^2	20	10^4	10^6	10^7	10^9	7
3	24, 48, 72, 96	10^3	6×10^2	10^2	5	0	0	0	0	More than 3 mo
4	24, 72	10^3	10^2	10^3	50	10^3	10^7	10^9	—	6
5	24 only	10^3	10	10^5	10^7	10^8	10^9	—	—	5
6	24 only, dosage doubled	10^3	10^2	10^2	10^3	10^5	10^6	4×10^8	10^9	7
Controls		10^4	10^5	10^8	10^9	—	—	—	—	3

* Per ml of blood.

RESULTS

In vitro effects of drugs

Soon after inoculation into cell cultures the trypanosomes seemed to clump in between the cells. Parasites increased about 2-fold every 48 hr; an inoculum of 10^3 produced about 10^7 parasites in 2 days (Table I). Cultures initiated with 24-hr-old trypsinized cells seemed to grow better. Also cultures were initiated from either the blood of mice (3 days postinfection), or from bloodstream trypomastigotes in established cultures.

The trypanosome cultures were treated with various concentrations of metronidazole and chloroquine (Table I) 48 hr postinoculation. Metronidazole at 0.003 mg/ml and chloroquine at 0.0024 mg/ml concentration did not kill the parasites, but there was no apparent increase in number. Also, they were not infective when inoculated into mice (Table I). However, at levels above these concentrations (Table I), the parasites were dead within 24 hr, whereas at those below these concentrations (metronidazole 0.0002 mg/ml; chloroquine 0.0012 mg/ml), the parasites multiplied and were infective. The controls (no drugs) remained infective.

Parasites from the treated cultures had more granulated cytoplasm than those from controls (no drugs).

In vivo effects of drugs

Infected mice that were inoculated with a single dose of chloroquine (0.08 mg/kg body weight) at 24 hr after infection had lower parasitemia and survived longer than those treated at 48 and 72 hr (Table II). Similar results were obtained with mice inoculated with a single dose of metronidazole (0.1 mg/kg body weight) or with a single dose of a combination of the 2 drugs (Table II).

Infected mice given multiple doses of each of the drugs survived longer than those given a single dose (Table III). However, the drugs administered individually only controlled the infection for a period but did not effect a cure, and the mice died from massive parasitemia.

No parasites were seen in the peripheral blood (wet mount examinations) of infected mice after 4 days if they were inoculated with 4 consecutive daily injections of the drug combination (Group 3; Table IV). The treatment was initiated 24 hr after infection and the absence of trypanosomes was confirmed 3 mo later using the haematocrit centrifuge technique (Woo, 1970) and by the intraperitoneal subinoculation of blood into naive mice. Mice that were treated with other dose regimens (e.g., 2 or 3 injections) died from the infection although they survived longer than the controls (Table IV).

DISCUSSION

This study demonstrated that *T. b. brucei* was sensitive to chloroquine and metronidazole. The *in vitro* effects of the drugs were very rapid; the motility of the parasite was reduced within minutes and all the trypanosomes were dead within 24 hr. Metronidazole is used to treat fungal infections, amoebiasis, giardiasis, and trichomoniasis in humans (James and Gilles, 1985). It was also effective in experimental *Trypanosoma cruzi* infection (Raether and Seidenath, 1983) and in combination with emetine was curative for malaria (James, 1985). The present study showed that the drug was effective against *T. b. brucei* and, in combination with chloroquine, cured trypanosome infection in mice. Chloroquine is the drug of choice for treatment and prophylaxis

against malaria and arthritis (Goodman and Gilman, 1980). It is also used in the treatment of other parasitic infections, e.g., hepatic amoebiasis (James and Gilles, 1985). Duch et al. (1980) suggested that all the substituted 4-amino-quinolines (of which chloroquine is a member) may have trypanocidal activity. Consequently, we were not surprised that chloroquine was effective against trypanosomes *in vitro*. At the concentrations that we used in our *in vitro* study, we did not notice any obvious effects on our feeder layer cells (B.H.K.).

Neither drug individually was effective in eliminating the parasite in mice at the dose levels (metronidazole 0.08 mg/kg body weight; chloroquine 0.1 mg/kg body weight) we used. However, they prolonged the survival time of the treated animals. Perhaps cure could be achieved with the individual drugs if the dosages used were larger and administered daily for a longer period. Although the 2 drugs administered individually were subcurative, they were curative when injected together daily for 4 days. This seemed to indicate an additive and/or synergistic effect of the 2 drugs. It should be noted that our findings are restricted to early treatment of a monomorphic infection. This drug combination, like suramin, may not be as effective when there is central nervous system (CNS) involvement because chloroquine is not known to cross the blood/brain barrier, whereas there is some penetration with metronidazole. Further experimental studies with chronic *T. brucei* infection (with CNS involvement) are planned.

Metronidazole inhibits DNA synthesis and causes degradation of DNA (Edwards et al., 1973; Knight et al., 1978). Chloroquine, on the other hand, has cytotoxic effects (Peters and Richards, 1983), including inhibition of nucleic acid synthesis, and also binds to DNA. It is likely that this interference with DNA synthesis by both metronidazole and chloroquine inhibited multiplication of the trypanosomes in our cultures. Chloroquine is known to inhibit glycolysis in bacteria (Fitch et al., 1984). Because the monomorphic trypanosomes depend on glycolysis for energy production, the chloroquine may have affected our monomorphic strain by inhibiting this pathway.

It is interesting to note that the drug dosages that we used (0.08 mg/kg body weight for chloroquines; 0.1 mg/kg body weight for metronidazole) in mice, if effective in humans, will result in a very low chemotherapeutic dose (e.g., for a 70-kg man, it will be 5.6 mg of chloroquine and 7 mg of metronidazole). In humans both drugs are also given orally because absorption is rapid and almost complete (James and Gilles, 1985); consequently, oral route of treatment would also be considered in future studies. The prescribed adult human dosage for metronidazole in the treatment for amoebiasis is 500 mg every 8 hr and with repeated administration for 3–4 days to provide a mean minimal plasma level of 12 μg/ml. The drug is relatively free of side effects and toxicity and is normally considered a safe drug at the recommended dosage. For treatment against malaria the daily prescribed adult dosage of chloroquine is 300 mg of the base. Steady plasma levels of about 125 μg/L are reached over 5 days and a plasma concentration of about 30 μg/L is sufficient for effective prophylaxis (Goodman and Gilman, 1980). The drug is normally well tolerated at the prophylactic dose regimen; however, side effects are common when curing clinical attacks.

Because the side effects and toxicity of the drugs are known and the drugs are commonly used and readily available in the tropics, we recommend that they be examined more closely for their additive and/or synergistic effects in the management of trypanosomiasis. It is premature at this stage of our study to suggest that the drug combination (metronidazole and chloroquine) and treatment regimen that we used for mice will be effective in humans for the treatment of African trypanosomiasis; however, this area of research may prove rewarding and warrants further study.

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DIVERSITY OF THE PARASITE ASSEMBLAGE OF *FUNDULUS ZEBRINUS* IN THE PLATTE RIVER OF NEBRASKA

John Janovy, Jr. and E. L. Hardin
School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0118

ABSTRACT: Changes in the values of the Shannon H′ diversity index as determined for individual hosts (infraassemblage diversity), host samples (sample assemblage diversity), and for species density are reported for an assemblage of 7 parasites in *Fundulus zebrinus* in the Platte River in Nebraska for a 5-yr period. The parasites were: *Myxosoma funduli* (gill), *Trichodina* sp. (gill), *Gyrodactylus bulbacanthus* (gill), *Salsuginus* sp. (gill), *Gyrodactylus stableri* (body surface), and *Neascus* sp. (=*Posthodiplostomum*; eyes and body cavity). In addition, relative abundance and equitability are given for each of the study years. Mean infraassemblage diversity, sample assemblage diversity, species density, and equitability were all significantly negatively correlated with river streamflow (measured in cubic feet per second) of the year prior to the sample, but were independent of the concurrent year's streamflow. Over the long term, *M. funduli* and *Trichodina* sp. were the most, and *G. bulbacanthus* was the least, abundant. Species pair prevalence and relative density correlations showed few long-term patterns of co-occurrence or microallopatry. The strongest association was between *M. funduli* and the *Neascus* sp. and was attributed to similarities in ecological requirements of intermediate hosts.

Previous papers on the parasites of *Fundulus zebrinus* (Pisces: Cyprinodontidae) in the Platte River in Nebraska have concerned parasite population dynamics, geographic distribution, and single year collection diversity (Knight et al., 1977, 1980; Adams, 1985, 1986; Janovy and Hardin, 1987). This paper deals with parasite species diversity over a 5-yr period and a wide range of river conditions. Informal observations, as well as the Adams (1985, 1986) work, suggested that the *F. zebrinus* parasite assemblage might be a good one to use to address questions about the dynamic behavior of groups of parasite species in a single host species. The Platte River streamflow fluctuates substantially over both annual and sometimes monthly periods. These fluctuations produce corresponding differences in the river's physical structure (see Janovy and Hardin, 1987, for study site description and individual parasite species' population dynamics). The Platte thus provides a natural experiment in which one can observe the response of a parasite assemblage to major environmental changes with which the host, of necessity, must deal.

THE HOST-PARASITE SYSTEM

The system studied consisted of a single host species, *F. zebrinus*, and the following parasite species or ecotypes: *Myxosoma funduli* Kudo, 1918 (Myxozoa: Myxosporea)—gill tissues; *Trichodina* sp. (Ciliophora: Peritrichia)—gill surfaces; *Salsuginus* sp. (Monogenea; ="*Urocleidus fundulus*" as reported in Adams, 1985, 1986; Janovy and Hardin, 1987)—gill surfaces; *Gyrodactylus bulbacanthus* Mayes, 1977 (Monogenea)—gill surfaces; *Gyrodactylus stableri* Hathaway and Herlevich, 1973 (Monogenea)—body surface, mainly fins; *Neascus* sp. (*Posthodiplostomum*: Digenea)—eye; and *Neascus* sp. (*Posthodiplostomum*: Digenea)—body cavity. The larval Digenea are termed eye and body cavity *Neascus*, respectively, in the following Results and Discussion. Although there were no structural differences that would allow distinction between larvae from the 2 infection sites, they are treated as separate ecotypes because of the possibility that eye infections affected host survival in a manner different from body cavity infections. In this study, observations were limited to the above 7 parasites because it was felt they represented several taxonomic groups as well as a range of life cycle types and transmission mechanisms. In 12 yr of study of *F. zebrinus* in the Platte River, no intestinal helminths have been discovered in this fish. *Scyphidia* sp. and *Lernea cyprinacea* are rare, the former especially so, and the latter occurs in a variety of Platte River fishes usually immediately following spring floods (Adams, 1985a). Thus, the questions approached in this study concerned behavior of a defined assemblage, rather than the diversity of an incompletely known or yet to be described supraassemblage.

QUESTIONS

With respect to the above system, the following questions were considered: (1) How does di-

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versity vary over time and river conditions? (2) How do the assemblage species mix and relative abundance vary over time and river conditions? (3) Is assemblage diversity related to host demographic factors such as size and sex? (4) Are there patterns of parasite species co-occurrence or mutual exclusion revealed by analysis of assemblage dynamics?

MATERIALS AND METHODS

Terminology is derived from Margolis et al. (1982). Study site, fish collection, and examination were as described previously (Janovy and Hardin, 1987). The data in this paper are from the same collections as reported earlier plus an additional sample from 9 June 1983; n = 21. Low-water years are 1982, 1985, and 1986; high-water years are 1983 and 1984. South Platte River annual average daily discharge rates at North Platte, Nebraska, in cubic feet per second, are for calendar year/water year, respectively: 1981—162/161; 1982—171/166; 1983—2,461/2,316; 1984—1,972/1,661; 1985—745/1,143; 1986—855/730. Water year 1982 ends on 30 September 1982 and water year 1983 begins on 1 October 1982. These streamflow averages are somewhat different from those reported earlier (Janovy and Hardin, 1987) for 2 reasons: (1) review of 1985 and 1986 records by Nebraska Department of Water Resources (NDWR) prior to official publication, and (2) misinterpretation of prepublication printouts by Janovy, an error that was not corrected until after the Journal of Parasitology, Volume 73, Number 4, had gone to press. Records for the North Platte gauging station, reference #06765500, are available for a small fee from the Nebraska Department of Water Resources, 301 Centennial Mall South, Lincoln, Nebraska 68508. The revisions in streamflow data reported here do not change either the previous results or the interpretation of them.

Diversity of the parasite assemblage was expressed by the Shannon H′ diversity index calculated using $\log_2 p_i$ at 2 levels, by species density, and by calculation of Lorenz equitability indices (Pielou, 1966a, 1966b; Hair and Holmes, 1975; Esch et al., 1979; McCauliffe, 1984; Adams, 1986). Infraassemblage diversity (H'_{IA}) was calculated using minus sum $p_{ij}\log_2 p_{ij}$ where p_{ij} is the proportion of parasite species i in host j. H'_{IA} values for a single day's collection were averaged to obtain a mean H'_{IA}, or mean infraassemblage diversity. Sample assemblage diversity, or H'_{SA}, was calculated using minus sum $p_{ik}\log_2 p_{ik}$ where p_{ik} is the proportion of species i in sample k. Species density was expressed as the average number of parasite species per host individual, calculated for each fish sample, and is the mean of the frequency distribution obtained by plotting number of individual hosts against parasite species/host classes.

The significance of H'_{IA}, mean H'_{IA}, H'_{SA}, and species density differences were assessed using 1-way single variable analysis of variance with individual fish H'_{IA} being the variable and sample being the group, in the case of infraassemblage diversity, and sample means being the variable and year being the group, in the cases of mean H'_{IA}, H'_{SA}, and species density. Chi-square goodness-of-fit, Bartlett's, and Cochran's tests were performed to test for normality and homogeneity of variances, with the probability of rejecting a true null hypothesis being taken as 0.05.

Evenness of parasite species representation was assessed using the Lorenz equitability index, obtained by calculating the area under a polygon resulting from the plot of proportionate accumulation of species (ordinate) against proportionate accumulation of individuals (abcissa), and multiplying that area times 2 (McAuliffe, 1984). Correlations between mean H'_{IA}, H'_{SA}, species density, and Lorenz equitability, and streamflow values were also determined.

Host demographic classes were compared using regression and *t*-test statistics. The least squares line and correlation coefficient were calculated for the total collection of 468 fish using host length vs. infraassemblage diversity. H'_{IA} values were also compared by *t*-test for the different sexes within streamflow conditions and for the same sex between high- and low-water years. Between-sex/between-water condition comparisons were not considered to be of biological significance.

Co-occurrence was assessed by calculation of prevalence and relative density correlations, in which relative density is defined as average number of parasites per fish (infected + uninfected) in a sample (Margolis et al., 1982). Prevalences and relative densities in high- and low-water year samples, and in all 23 collections, were used to calculate correlation coefficients for the 21 species pair combinations.

Statistical methods are those presented in Dixon and Massey (1957) and Steel and Torrie (1980).

RESULTS

Changes in the values of mean H'_{IA}, H'_{SA}, and species density over time are shown in Figure 1. Results of analysis of variance using individual H'_{IA} values as variables and samples as groups indicated there were no significant differences between mean H'_{IA} for the 23 samples ($F[22,445] = 0.4233$, P [greater F by chance alone] = 0.75). However, similar analysis using means as variables and years as groups, indicated there were significant differences between mean H'_{IA} values between years ($F[4,18] = 15.2337$, P [greater F by chance alone] < 0.001), between H'_{SA} between years ($F[4,18] = 3.6600$, P [greater F by chance alone] < 0.05), and between species densities between years ($F[4,18] = 113.3300$, P [greater F by chance alone] < 0.001). Both mean H'_{IA} and H'_{SA} using years as groups passed Bartlett's test for homogeneity of variances ($F[4,286] = 1.6870$ and 1.7480, respectively). The variances of both H'_{IA} using samples as groups, and species densities, failed Bartlett's test but passed Cochran's test.

In all 23 samples of H'_{IA}, the variance was less than the mean, but 15 of the 23 were not normally distributed as revealed by chi-square tests (alpha = 0.05). The mean (variance) values for H'_{IA} were: 1.05 (0.47) for all fish (n = 468), 1.14

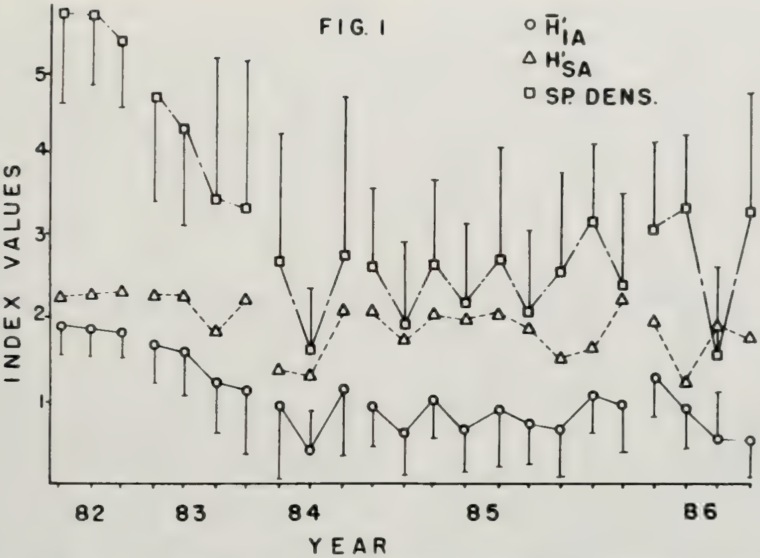

FIGURE 1. Changes in the values of mean infraassemblage diversity ($\bar{H}'_{IA}$), sample assemblage diversity (H'_{SA}), and species density (SP. DENS.) of the parasite assemblage of *Fundulus zebrinus*. Collection dates and sample sizes are: 1982—5/20 (16), 7/19 (16), 8/02 (26); 1983—6/09 (21), 6/15 (11), 7/14 (21), 7/25 (27); 1984—6/01 (19), 6/19 (15), 7/25 (8); 1985—5/20 (23), 5/25 (27), 6/08 (24), 6/15 (24), 6/27 (19), 6/29 (22), 7/01 (22), 7/20 (22), 8/10 (19); 1986—5/21 (24), 6/04 (24), 6/29 (24), 7/16 (14). Bar = standard deviation.

(0.58) for high-water years (n = 119), and 1.02 (0.44) for low-water years (n = 349). None of the 3 distributions were normally distributed as determined by chi-square tests, mainly because of the large zeroth classes.

Lorenz equitabilities for the 5 yr, for high- and low-water conditions, and for the overall study, as well as order of abundance for the 7 parasite types, are given in Table I. The numbers indicate that over the long term, *Myxosoma funduli* and *Trichodina* sp. are among the most abundant, and that *G. bulbacanthus* is among the least

TABLE I. *Order of abundance, relative abundance, and Lorenz equitability for the various species in the parasite assemblage of* Fundulus zebrinus.

Year	Parasites in order of abundance* (relative abundance)							Lorenz equitability
1982	T (0.37)	M (0.22)	BCM (0.14)	GS (0.12)	EM (0.11)	U (0.03)	GB (0.01)	0.582
1983	BCM (0.28)	T (0.24)	EM (0.22)	M (0.16)	GS (0.06)	U (0.03)	GB (0.01)	0.616
1984	T (0.46)	M (0.31)	U = GS (0.07)	GS = U (0.07)	EM (0.05)	BCM (0.03)	GB (0.01)	0.451
1985	M (0.30)	T (0.28)	GS (0.27)	U (0.10)	GB (0.04)	BCM (0.008)	EM (0.002)	0.525
1986	T (0.6)	U (0.17)	GS (0.11)	M (0.06)	EM (0.02)	BCM (0.01)	GB (0.01)	0.359
High water	T (0.28)	BCM (0.23)	M (0.19)	EM (0.18)	GS (0.17)	U (0.04)	GB (0.02)	0.774
Low water	T (0.40)	M (0.21)	GS (0.17)	U (0.09)	BCM (0.06)	EM (0.05)	GB (0.02)	0.551
Overall	T (0.37)	M (0.20)	GS (0.14)	BCM (0.10)	EM = U (0.08)	U = EM (0.08)	GB (0.02)	0.612

* Abbreviations: M = *Myxosoma funduli*, T = *Trichodina* sp., GB = *Gyrodactylus bulbacanthus*, GS = *G. stableri*, U = *Salsuginus* sp., EM = eye *Neascus*, BCM = body cavity *Neascus*.

TABLE II. *Correlations between annual streamflow and sample diversity, species density, and equitability for the parasite assemblage of* Fundulus zebrinus.*

	CY streamflow	WY streamflow	Previous CY flow	Previous WY flow	Mean H'_{IA}	H'_{SA}	Species density	Equitability
CY streamflow	1.00	0.94	−0.10	−0.09	−0.09	−0.08	−0.08	−0.08
WY streamflow		1.00	−0.09	0	−0.17	−0.04	−0.20	−0.08
Previous CY flow			1.00	0.97†	−0.70†	−0.45†	−0.71†	−0.48†
Previous WY flow				1.00	−0.79†	−0.55†	−0.77†	−0.58†
Mean H'_{IA}					1.00	0.60†	0.95†	0.67†
H'_{SA}						1.00	0.52†	0.98†
Species density							1.00	0.58†
Equitability								1.00

* n = 23. r values outside the range ±0.415 are high enough to reject the hypothesis that $r = 0$ at the 5% level and are indicated in the above table by a dagger (†).

abundant, but that the relative positions of the other assemblage members vary with year and water condition.

Correlations between annual streamflow, mean infraassemblage diversity, sample assemblage diversity, species density, and equitability for the 23 samples are given in Table II. In no case was the correlation coefficient between a diversity indicator value and current calendar or water year streamflow high enough to reject the hypothesis of independence. However, in all cases the correlation coefficients between diversity indicators and previous calendar or water year streamflows were high enough to reject the independence hypothesis at the 0.05 level.

Diversity of the *F. zebrinus* parasite assemblage was not related to host demographic factors. In 4 of the 23 samples, H'_{IA} values for males were significantly higher than those of females; in 8 of the 23, the reverse was true; and in the remaining samples there were no significant differences between the H'_{IA} values of the 2 sexes. When samples were combined into high- and low-water year categories, no significant differences either between sexes within water conditions, or within sexes between water conditions, were revealed by t-tests. There was no correlation between host length and H'_{IA} in the entire collection of fish ($r = -0.04$; n = 468; regression line slope = -0.04, y-intercept = 1.27; $F[1,466] = 0.91$).

Prevalence and density correlations revealed few patterns of co-occurrence or microallopatry (Tables III, IV). Positive correlations between prevalences were generally low, except for the 2 *Neascus* types, as well as each of them with *M. funduli* (Table III). The latter correlation was stronger in low-water years than in high. Negative correlations were observed between the 2 gill monogenes *U. fundulus* and *G. bulbacanthus*, but only in low-water years was the r value high enough to reject the independence hypothesis at the 0.05 level.

Aside from the expected high r values for the eye *Neascus*/body cavity *Neascus* comparisons, there was little evidence that conditions affecting relative density of one species also affected that of another (Table IV). Positive correlations high enough to reject the independence hypothesis were observed between both *Neascus* types and *M. funduli*, but that relationship did not hold in high-water years. Significant positive correlations were also observed between the *Neascus* types and *Trichodina* species' relative densities under low-water conditions. No negative correlations, between relative densities, high enough to reject the independence hypothesis, were observed (Table IV).

DISCUSSION

This paper presents a detailed description of the long-term dynamic behavior of diversity and equitability indices as determined for a parasite assemblage in a single host species in a fluctuating environment. In general, the results suggest that diversity is influenced by environmental conditions through their effect on some, but not all, members of the assemblage. Thus, in this system the primary effect of high water was to reduce species density mainly through a reduction in the prevalence and relative abundance of larval Digenea (Fig. 1; see also Janovy and Hardin, 1987).

The streamflow effect, however, exhibits a lag, at least at the assemblage level. This out-of-phase effect is seen in the correlations in Table II, in which mean H'_{IA}, H'_{SA}, species density, and equitability are all independent of the concurrent

TABLE III. *Between-species prevalence correlations.**

Parasite species	*Myxosoma funduli*	*Trichodina* sp.	*G. bulbacanthus*	*Salsuginus* sp.	*G. stableri*	Eye *Neascus*	Body cavity *Neascus*
Overall prevalence correlations:							
M. funduli	1.00	0.45†	−0.31	0.21	0.09	0.75†	0.81†
Trichodina sp.		1.00	−0.01	0.57†	0.39	0.26	0.41
G. bulbacanthus			1.00	−0.39	0.39	−0.11	−0.12
Salsuginus sp.				1.00	0.27	−0.11	0.01
G. stableri					1.00	−0.13	−0.01
Eye *Neascus*						1.00	0.96†
Body cavity *Neascus*							1.00
High-water years:							
M. funduli	1.00	0.30	−0.46	0.59	0.13	0.50	0.69
Trichodina sp.		1.00	0.22	0.57	0.69	−0.23	0.23
G. bulbacanthus			1.00	−0.53	0.40	−0.03	−0.12
Salsuginus sp.				1.00	0.20	−0.39	−0.02
G. stableri					1.00	−0.03	0.29
Eye *Neascus*						1.00	0.87†
Body cavity *Neascus*							1.00
Low-water years:							
M. funduli	1.00	0.62†	−0.18	0.36	0.53†	0.81†	0.83†
Trichodina sp.		1.00	−0.15	0.57†	0.26	0.58†	0.59†
G. bulbacanthus			1.00	−0.56†	0.35	−0.03	−0.02
Salsuginus sp.				1.00	−0.13	0.25	−0.02
G. stableri					1.00	0.37	0.35
Eye *Neascus*						1.00	0.99†
Body cavity *Neascus*							1.00

* For overall prevalence correlations, n = 23 and r values outside the range ±0.415 are high enough to reject the hypothesis that $r = 0$ at the 5% level. For high-water years, n = 7 and the significant r values are ±0.754; for low-water years n = 16 and the significant r values are ±0.497. All correlations are between z-transformed (standardized) variables. r values high enough to reject the null hypothesis are indicated by a dagger (†).

TABLE IV. *Between-species density correlations.**

Parasite species	*Myxosoma funduli*	*Trichodina* sp.	*G. bulbacanthus*	*Salsuginus* sp.	*G. stableri*	Eye *Neascus*	Body cavity *Neascus*
Overall density correlations:							
M. funduli	1.00	0.30	0.24	−0.11	0.22	0.42	0.49†
Trichodina sp.		1.00	−0.05	0.35	0.34	0.40	0.33
G. bulbacanthus			1.00	−0.36	0.18	0.09	0.13
Salsuginus sp.				1.00	0.09	−0.17	−0.22
G. stableri					1.00	0.04	0.02
Eye *Neascus*						1.00	0.92†
Body cavity *Neascus*							1.00
High-water years:							
M. funduli	1.00	0.22	−0.18	0.83†	0.26	0	−0.02
Trichodina sp.		1.00	0.25	0.55	0	0.16	0.13
G. bulbacanthus			1.00	−0.19	0.70	0.37	0.40
Salsuginus sp.				1.00	0.27	−0.09	−0.07
G. stableri					1.00	0.40	0.27
Eye *Neascus*						1.00	0.96†
Body cavity *Neascus*							1.00
Low-water years:							
M. funduli	1.00	0.34	0.42	−0.14	0.42	0.58†	0.73†
Trichodina sp.		1.00	−0.15	0.32	0.32	0.62†	0.55†
G. bulbacanthus			1.00	−0.45	0.19	−0.11	0.07
Salsuginus sp.				1.00	−0.19	−0.02	−0.08
G. stableri					1.00	0.37	0.41
Eye *Neascus*						1.00	0.87†
Body cavity *Neascus*							1.00

* For overall density correlations, n = 23 and r values outside the range ±0.415 are high enough to reject the hypothesis that $r = 0$ at the 5% level. For high-water years, n = 7 and the significant r values are ±0.754; for low-water years n = 16 and the significant r values are ±0.497. All correlations are between z-transformed (standardized) variables. r values high enough to reject the null hypothesis are indicated by a dagger (†).

year's streamflow and significantly negatively correlated with the previous year's streamflow. This effect is seen most clearly in the larval Digenea, in which relative abundance and prevalence remain high during 1983, a high-water year, falling only in the second consecutive high-water year (Table I and Janovy and Hardin, 1987).

Species pair prevalence and relative density correlations suggest that apparent associations between assemblage members, whether negative or positive, are likely of ecological origin. The major evidence supporting this conclusion is the difference in r values between high- and low-water conditions. For example, significant positive correlations between *M. funduli* and the *Neascus* types, in low-water years, could result from similarities in the ecological requirements of intermediate hosts. The tubificid oligochaetes that have been implicated in the life cycle of *Myxosoma* species require a microhabitat similar to that of snails (Markiw and Wolf, 1983). In low-water years, especially the second and third of a row, shallow warm pools conducive to the buildup of snail and oligochaete populations are present much of the summer. In high-water years these pools are absent and the *Neascus* intermediate hosts are rare and widely dispersed (unpubl. field notes).

The physical structure of the South Platte River is a function of streamflow. At low water, the river is a relatively complex braided stream with numerous different microhabitats such as pools, rivulets, etc. At high water, this complexity is lost. The significant negative correlations between diversity, species density, and equitability, and the previous year's streamflow (Table II) suggest that heterogeneity in the host's physical environment promotes diversity in the parasite assemblage. The explanation for this relationship probably lies in the extent to which river heterogeneity also promotes survival of a variety of parasite intermediate or infective stages.

This paper adds to the Adams (1986) single year's work in 2 ways: it provides a long-term picture of the assemblage dynamics, and it involves parasites in addition to those on the gills. Adams (1986) also claimed that diversity of the gill fauna was independent of host size and sex, an observation which the present paper extends to the entire assemblage over the long term. It also complements the Kennedy et al. (1986) paper in that it compares a number of transmission mechanisms in a single host species, instead of a single transmission mechanism, namely ingestion, in a number of species. Thus, it is not surprising that Kennedy et al. (1986) attributed differences between intestinal helminth assemblage diversities to breadth of host feeding niches, whereas in the present study such differences are attributed ultimately to heterogeneity in the host's physical environment.

The data in this paper are clearly hierarchical on 2 dimensions, host population and time, in that they are derived from observations on single fish, single samples, annual and multiyear collections. The single host species provides a natural organizational unit in this system, and can probably be thought of as a holon in the Koestler sense (Koestler, 1967), as well as a practical study unit of the prairie river ecosystem (see O'Neill et al., 1986). Knowledge of parasite transmission mechanisms, the local biology of intermediate hosts, and population ecology of the fish would furthermore provide estimates of the "connectedness" of various parts of this system (Fahrig et al., 1983).

The field observations reported here show that the distribution of individual H'_{IA} values are remarkably close to those predicted by Bowman et al. (1971) using moments of exact formulas and Monte Carlo simulations, except that the values for the *F. zebrinus* parasite assemblage include a fairly large zeroth class. Bowman et al. (1971) gave theoretical values of H' (variance H') of 1.232 (0.03) and 1.731 (0.03) for samples of 20 from communities of 5 and 10 species, respectively, and 1.322 (0.004) and 1.923 (0.006) for samples of 100 from the same communities. Thus, the major departure from the theoretical model, i.e., random selection from a parent community in which the species are not equally represented, is in the number of individual hosts having 1 or no species of parasites.

This difference may reflect the mechanism by which hosts "sample" the supraassemblage. In the present study, for example, probability of infection by a particular parasite species can be approximated by the prevalence of that species in a host sample. Thus, unlike the Bowman et al. (1971) model, prevalences lower than 100% allow for hosts that have not "successfully sampled" parasite suprapopulations. If the prevalence data reported in Janovy and Hardin (1987) are used to calculate the probability of a fish having 0 or 1 parasite species, then it is possible to calculate a theoretical number of fish whose infraassemblage diversity is 0. This was done for each of the 23 samples in this study, and the

results tested by chi-square for differences from the observed number of fish showing H'_{IA} values of 0. In only 1 of the 23 cases was the observed number of the zeroth-class fish significantly different from the theoretical prediction. This result suggests that parasite infraassemblage diversity might be predicted from a simple probability of infection model based on single species' prevalences.

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FUNCTIONAL RELATIONSHIPS AND MICROHABITAT DISTRIBUTIONS OF ENTERIC HELMINTHS OF GREBES (PODICIPEDIDAE): THE EVIDENCE FOR INTERACTIVE COMMUNITIES

T. M. Stock and John C. Holmes
Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

ABSTRACT: The significance of interspecific interactions as a structuring force in the enteric helminth communities of 4 species of grebes (*Aechmophorus occidentalis, Podiceps grisegena, P. nigricollis,* and *P. auritus*) was evaluated. Patterns of microhabitat distribution revealed that helminths resided in predictable locations along the length of the small intestine. Individual species of helminths occupied a high proportion of the intestine and varied in position in different host species suggesting a broad tolerance for conditions along the intestine. However, in individual birds, helminths were much more restricted in distribution, overlapped considerably less than their overall ranges would suggest, congeners had complementary distributions, and there was evidence of interference by at least 1 core species, all suggesting that interactions are important in these communities. Nevertheless, vacant niches were present in most birds, and core species were not distributed more evenly than would be expected through random placement. This suggests that important resources may not be distributed evenly along the intestinal gradient.

Many parasites are known to occupy precise, predictable, and limited locations within their hosts (see reviews in Crompton, 1973; Holmes, 1973; Crompton and Nesheim, 1976). Price (1980, 1984) argued that the generality of this observation was the consequence of highly specialized organisms exploiting complex environments with steep gradients in environmental conditions or resources. A group of such species, each adapted independently to a restricted location in a host, would form an isolationist community.

An alternative explanation for the site specificity demonstrated by parasites was limitation of microhabitat distribution mediated by interspecific interactions (Holmes, 1973; Bush and Holmes, 1986). A group of species whose distributions were limited in this way would form an interactive community. Holmes and Price (1986) contrasted these 2 types of communities and presented several attributes that could be used to differentiate isolationist from interactive assemblages.

The basic difference between these views of communities obviously centers on whether the realized distributions of the component species are limited primarily by the degree of specialization or by the presence of other species. The degree of specialization of intestinal parasites can be considered to be the inverse of their physiological tolerance to the range of conditions present in different regions of the intestine. This range of tolerance is analogous to Hutchinson's (1957) concept of fundamental niche. The best measure of a helminth's fundamental niche would be its potential distribution in controlled monospecific infections; in the absence of such data, the fundamental niche may be estimated by the distribution of the species summed across all birds in which it occurred (Bush and Holmes, 1986). Realized niches, the ranges actually occupied in individual birds, are subsets of the fundamental niches. Presumably, realized niches represent the optimal part of the fundamental niche (where infrapopulations are small) or the portions of the fundamental niches actually available to species subjected to interactions with other helminths (Pianka, 1975).

Because isolationist communities are assumed to be unsaturated aggregations of specialists, fundamental niches should be small and similar in size to realized niches, and vacant niches (unoccupied regions) should be common in individual birds. Niche breadths may increase with increasing populations, but there should be little or no evidence of interactions (such as displacement); overlaps between species should increase with increases in combined population size. Finally, enteric helminth species should be distributed along the intestine either at random or according to the quality of the habitat.

Conversely, interactive communities should include species that are more generalized in microhabitat use. Fundamental niches should be relatively broad, and realized niches should be reduced in the presence of other similar species. Interactive communities are assumed to be sat-

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urated and to have few vacant niches. Niches should be sensitive to variations in population size but overlaps with other species should be avoided. The core species (species that are common and abundant) should be distributed more evenly along the intestine than expected by chance (Hanski, 1982), and congeners should be complementary in their use of the habitat (Brown, 1975; Pianka, 1975).

Stock and Holmes (1987a) showed that 3 of 4 species of grebes (Podicipedidae) had infracommunities with high proportions of regularly cooccurring core species. These were present in reasonably high populations and conferred a high degree of predictability to the communities. In this paper, we will examine the functional relationships and the patterns of microhabitat distributions of core species in order to evaluate the importance of interaction and specialization as forces responsible for maintaining intestinal helminth community structure in grebes.

MATERIALS AND METHODS

Adult grebes of 4 species, *Aechmophorus occidentalis, Podiceps grisegena, P. nigricollis,* and *P. auritus,* were collected during the breeding season (May–September) on 9 lakes in Alberta by shooting (for details see Stock, 1985). Birds were weighed, and intestinal tracts were removed and quick frozen by flooding with ethanol cooled to −70 C with solid carbon dioxide. Later in the laboratory, each intestine was partially thawed and its length measured. The small intestine was divided into 20 equal sections, each of which was placed in a separate vial and maintained frozen. Later, each was examined individually; the intestinal wall was split, the mucosa scraped, and the number of each species of helminth determined. For tapeworms, unless indicated otherwise, the position of the scolex was considered to represent the position of the individual. The large intestine and caeca were also stored and examined separately. See Bush and Holmes (1986) for details of these procedures.

If preliminary estimates suggested more than 500 individual helminths in a section, then large-bodied species, nematodes, and trematodes were removed and individually counted and 2 10% subsamples were removed and counted to estimate the number of small-bodied cestodes in the section. The locations of the median, anteriormost, and posteriormost individuals of each species of helminth in each small intestine were transformed from a discrete (section number) to a continuous variable (percent of the length of the small intestine; pylorus = 0%, ileocaecal junction = 100%). Within a section, individuals were assumed to be equally spaced, with the space between the end of the segment and the first (or last) individual assumed to be one-half of the distance between individuals. Distance measures were calculated by a FORTRAN program written by Drs. J. F. Addicott and J. C. Holmes. Overlaps were calculated using percent similarity (equation 1 of Hurlbert, 1978) by an APL program written by Mr. N. Weimer. Representative helminth specimens were submitted to the USNM Helminthological Collection, accession numbers 79321 to 79343 (see Stock and Holmes, 1987a, for specific assignations).

RESULTS

General patterns of distribution

The sizes of the intestines of grebes varied among species (Table I). There was almost a 3-fold difference in intestinal length between the smallest *Podiceps nigricollis* and the largest *Aechmophorus occidentalis.* Mean lengths ranged from 139 cm for *A. occidentalis* to 99 cm for *Podiceps auritus.* Coefficients of variation of intestinal length were greater for the 2 large grebes (*A. occidentalis* and *Podiceps grisegena*) than for the 2 small grebes (*P. nigricollis* and *P. auritus*). That this greater variation is not due to differential contraction in the more muscular foreguts of large grebes is indicated by the similarity in location of a midintestinal marker, Meckel's diverticulum, in these birds. Similar coefficients of variation of the position of this feature in large and small grebes also suggest that differential contraction is not important. The greater variation in intestinal length may be due to the greater variation in body size in the large grebes, especially *A. occidentalis* (Table I). Despite the differences in intestinal size, the relative positions of characteristics such as the pancreas, its ducts, and Meckel's diverticulum were similar (Table I). Therefore, locations expressed as a percent of small intestine length are comparable among grebes.

Parasites can potentially inhabit all 20 sections of the small intestine in all grebes; no sections were always unoccupied. Large grebes had higher proportions of unoccupied sections than small grebes, with a maximum of 13% in *A. occidentalis.* The distribution of vacant sections was not random, but increased toward the ileocaecal junction. For all grebes, the last intestinal section was the most frequently unoccupied (Fig. 1).

All 4 grebe species were characterized by a reduction in species richness (mean number of species of helminths per section) toward the posterior end of the intestine (Fig. 2). Maximum species richness for *P. nigricollis* occurred in the midintestine and for *P. grisegena* in the anterior quarter. For *A. occidentalis* there was little change over the anterior ¾ of the intestine, and the pattern in *P. auritus* was bimodal with peaks in the duodenal region and at 75% of the length of the

TABLE I. *Selected morphological characteristics of the adults of 4 species of grebes.*

	Aechmophorus occidentalis (n = 20)	*Podiceps grisegena* (n = 33)	*Podiceps nigricollis* (n = 31)	*Podiceps auritus* (n = 7)
Body weight, g (SD)	1,262 (247)	1,124 (159)	366 (49)	393 (43)
CV %*	20	14	13	11
Intestine length, cm (SD)	139.2 (19.7)	118.4 (14.9)	104.1 (14.0)	99.4 (4.3)
CV %	14	13	4	4
Range in intestine length, cm	108–205	88–150	72–138	94–107
Mean section with Meckel's diverticulum (SD)	11.4 (0.5)	11.2 (0.9)	10.7 (0.5)	10.7 (1.0)
CV %	4	8	5	9

* Coefficient of variation, 100 × SD/mean.

intestine. *Aechmophorus occidentalis* and *P. auritus* tended to have fewer species per section throughout the intestine than *P. grisegena* and *P. nigricollis.* Coefficients of variation for all grebes increased from anterior to posterior, indicating that the latter region was the least predictable with regard to species richness.

Three grebes, *A. occidentalis, P. grisegena,* and *P. auritus,* had the same pattern of habitat use by parasites as measured by mean number of worms per section (Fig. 3). Maximal values occurred in the duodenal region followed by a gradual decline toward the posterior end. In *P. nigricollis,* secondary peaks in the mid- and posterior intestine were superimposed on this pattern. As in the case of species richness, coefficients of variation increased from the anterior to the posterior part of the intestine; they were especially high in the posterior sections in *P. nigricollis.* The posterior part of the intestine was the least predictable in terms of the numbers of both species and worms per section.

One measure of the location of a species of helminth is the position of its medianth individual in each host. The mean of these positions is a measure of the usual location of that species (Tables II–V). Frequency distributions of mean

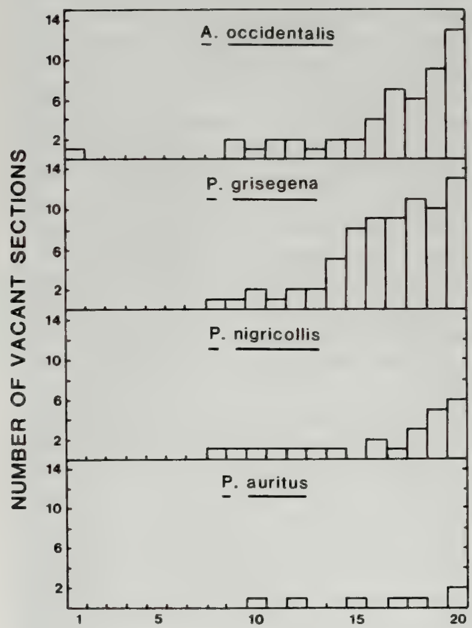

FIGURE 1. Frequency of vacant sections in intestines of 4 grebes.

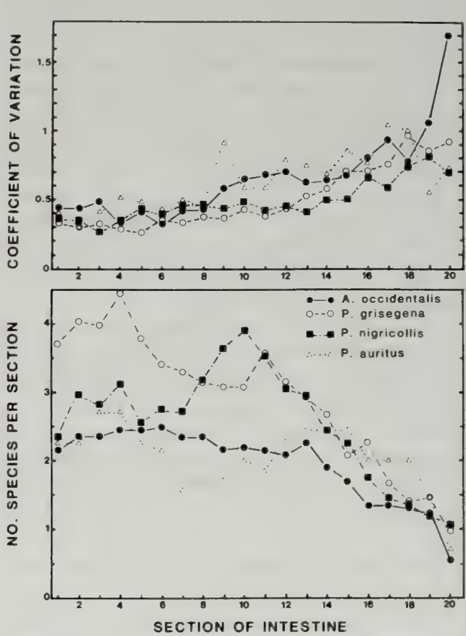

FIGURE 2. Mean number of helminth species per intestinal section, with coefficients of variation, in 4 grebes.

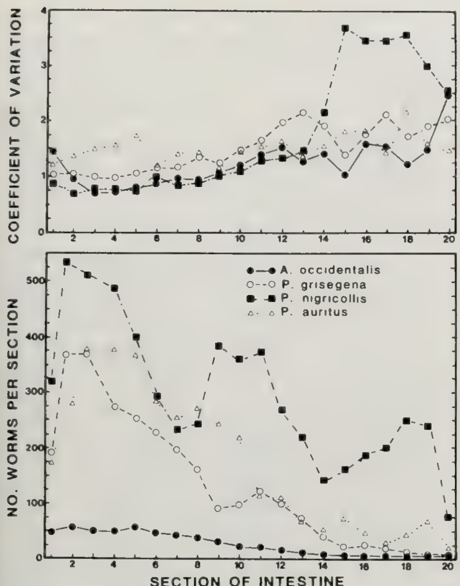

FIGURE 3. Mean number of worms per intestinal section, with coefficients of variation, in 4 grebes.

positions in each host species indicated that the species of helminths were not concentrated in (nor absent from) any particular part of the intestine. Chi-square tests indicated that the numbers of species in each quarter of the intestine did not differ from uniform distributions (*P. nigricollis*, χ^2 (3 df) = 6.37, 0.10 > P > 0.05; other grebes $\chi^2 < 1.2$, $P > 0.5$). Therefore, the patterns in species richness and numbers of worms described above are not due to differences in the numbers of species adapted to live in different portions of the intestine.

Bush and Holmes (1983) proposed a method using MacArthur's (1957) broken-stick model to test the distribution of species in individual infracommunities. This test provides a quantitative technique for determining if the positions of species distributed along a gradient are clumped, randomly distributed, or distributed more evenly than would be expected by chance alone. The null hypothesis of independent, random distribution could not be rejected for data from infracommunities in *A. occidentalis* (12 infracommunities above, 8 below predicted values), *P. auritus* (3 above, 4 below), and *P. nigricollis* (14 above, 17 below). However, data from infracommunities in *P. grisegena* (25 above, 8 below predicted values; $Z = 3.13$, $P < 0.05$) indicated a clumped distribution. The distance between the position of the posteriormost species and the posterior end of the intestine was the largest segment in 14 of 33 (42%) of the *P. grisegena* infracommunities. This suggests that the broken-stick model may not be appropriate in this instance.

Specific distributions

Locations of the common species (core and secondary or recurrent group and associate species; see Stock and Holmes, 1987a) in each

TABLE II. *Locations of intestinal helminths in 20* Aechmophorus occidentalis *from Alberta, Canada. Locations are expressed as percents of intestinal length. 0 = pylorus, 100 = ileocaecal junction, E = range expanded significantly (*P *< 0.05) with increasing populations.*

Helminth	No. infected	Mean intensity	Mean position (SD)	Mean anterior (SD)	Mean posterior (SD)	Mean range (SD)	Total occupied range
Pararetinometra lateralacantha	1	2	2.5	1.3	3.8	2.5	3
Contracaecum ovale	1	1	7.5	7.5	7.5	—	—
*Tylodelphys podicipina**	15	67	17.3 (9.6)	3.0 (5.7)	50.4 (19.8)	50.8 (18.4)	79
Tatria biremis	7	5	17.9 (11.6)	8.3 (4.5)	21.8 (14.8)	23.7 (14.7)	42
*Dubininolepis furcifera**	18	332	19.7 (6.5)	0.9 (1.8)	60.4 (19.7)	59.5 (19.8)	97
Diorchis sp. P	2	2	30.0 (24.8)	29.4 (25.6)	30.6 (23.9)	2.5 (0)	36
*Petasiger nitidus**	17	115	36.7 (18.2)	11.7 (16.7)	67.5 (23.1)	59.4 (20.5)	100
Apatemon gracilis	5	47	44.2 (29.4)	19.9 (27.8)	68.4 (40.2)	60.7 (32.5)	99
Tatria decacantha	1	3	52.5	47.5	57.5	10	10
*Wardium paraporale**	20	25	54.4 (19.7)	35.5 (17.6)	74.7 (23.2)	41.3 (17.2) E	92
Dubininoelpis podicipina	1	10	56.7	47.5	62.5	15	15
*Ligula intestinalis**	16	8	70.6 (9.6)	61.8 (10.4)	80.0 (10.6)	19.3 (11.1) E	47
Capillaria obsignata	2	3	80.0 (17.7)	67.5 (17.7)	92.5 (17.7)	—	25
Polymorphus marilis	2	2	85.0 (3.5)	84.4 (2.7)	85.6 (4.4)	2.5	6
Tetrabothrius immerinus	8	3	88.5 (6.5)	78.9 (21.7)	90.5 (6.7)	23.2 (21.9)	66
Schistocephalus solidus	6	6	91.1 (3.6)	88.1 (5.7)	94.2 (3.5)	9.1 (3.5) E	18

* Core species (see text).

TABLE III. *Locations of intestinal helminths in 33* Podiceps grisegena *from Alberta, Canada. Locations are expressed as percents of intestinal length. 0 = pylorus, 100 = ileocaecal junction, E = range expanded significantly (*P *< 0.05) with increasing populations, P = mean position moved posterior significantly (*P *< 0.05) with increasing populations.*

Helminth	Number infected	Mean intensity	Mean position (SD)	Mean anterior (SD)	Mean posterior (SD)	Mean range (SD)	Total occupied range
Pararetinometra lateralacantha	6	56	2.8 (0.5) P	0.2 (0.3)	8.0 (3.1)	7.8 (3.3) E	12
*Tylodelphys podicipina**	30	179	9.3 (5.8) P	1.2 (3.4)	30.2 (18.1)	30.0 (18.4) E	79
*Dubininolepis furcifera**	32	109	9.4 (3.5)	1.4 (2.4)	20.2 (8.8)	18.0 (8.6) E	44
*Tatria biremis**	28	377	9.7 (4.0)	1.3 (2.0)	23.8 (11.2)	22.5 (12.2)	47
Diorchis sp. O	1	3	13.8	11.3	17.5	6.3	6
Cotylurus sp.	1	1	22.5	22.5	22.5	—	—
*Schistotaenia srivastavai**	24	18	22.7 (5.2)	19.4 (6.2)	27.5 (6.9)	9.7 (8.9) E	33
Retinometra sp.	1	1	27.5	27.5	27.5	—	—
*Petasiger nitidus**	31	1,025	28.9 (6.6)	5.5 (10.8)	76.8 (19.5)	71.3 (28.7) E	100
*Tatria decacantha**	27	1,085	36.2 (15.5)	13.5 (16.4)	67.8 (15.3)	54.3 (25.4) E	94
Schistotaenia tenuicirrus	1	1	37.5	37.5	37.5	—	—
*Wardium paeaporale**	32	113	40.9 (19.5)	25.4 (14.0)	63.8 (21.6)	38.4 (21.5) E	94
Apatemon gracilis	5	2	49.5 (28.8)	38.3 (18.3)	51.8 (29.2)	16.9 (16.0)	80
*Contracaecum ovale**	26	21	54.3 (8.6)	43.8 (12.2)	66.9 (13.4)	25.0 (20.0)	91
*Dioecocestus asper**	25	2	54.4 (17.0)	45.4 (19.4)	57.5 (16.2)	13.2 (10.5)	76
Tetrabothrius immerinus	4	7	58.7 (42.9)	44.2 (31.8)	63.1 (45.1)	18.9 (16.2)	92
Immature strigeids	7	4	62.7 (33.0)	60.5 (33.9)	76.6 (27.4)	28.3 (37.3)	83
Ligula intestinalis	10	28	67.2 (26.5)	57.6 (28.0)	73.9 (29.5)	19.2 (21.2) E	95
*Capillaria obsignata**	29	21	70.9 (25.1)	43.4 (31.0)	79.8 (27.5)	46.3 (34.4) E	97
Schistocephalus solidus	13	49	82.7 (14.4)	62.4 (22.1)	91.9 (11.2)	32.0 (21.3)	84
Corynosoma constrictum	4	2	88.4 (6.6)	84.7 (9.0)	92.5 (0)	15.6 (0.9)	16
Polymorphus marilis	1	6	90.0	85.8	94.2	8.3	8

* Core species (see text).

TABLE IV. *Locations of intestinal helminths in 31* Podiceps nigricollis *from Alberta, Canada. Locations are expressed as percents of intestinal length. 0 = pylorus, 100 = ileocaecal junction, E = range expanded significantly (*P *< 0.05) with increasing populations, P = mean position moved posterior significantly (*P *< 0.05) with increasing populations.*

Helminth	Number infected	Mean intensity	Mean position (SD)	Mean anterior (SD)	Mean posterior (SD)	Mean range (SD)	Total occupied range
Pararetinometra lateralacantha	4	11	5.1 (2.4)	3.0 (3.9)	7.9 (0.7)	7.4 (0.2)	9
Tylodelphys podicipina	2	2	7.5	7.5	7.5	—	—
Trichobilharzia sp.	1	1	12.5	12.5	12.5	—	—
Nadejdolepis sp.	15	883	12.7 (3.5)	4.6 (3.6)	21.6 (11.0)	19.4 (12.9) E	47
Dubininolepis furcifera	7	61	12.8 (6.0)	4.1 (3.6)	24.2 (10.1)	20.1 (8.7)	42
Diorchis sp. O*	26	220	14.0 (9.6) P	3.5 (4.9)	27.6 (16.0)	26.1 (14.9) E	99
Schistotaenia srivastavai	2	2	15.0 (3.5)	14.4 (4.4)	15.6 (2.7)	2.5	6
*Tatria biremis**	31	2,794	18.1 (6.5)	0.1 (0.5)	51.5 (15.0)	51.4 (15.1)	97
Petasiger nitidus	5	44	20.0 (7.1)	8.7 (11.9)	35.7 (19.0)	27.0 (22.0) E	67
Schistotaenia colymba	16	7	20.9 (3.2)	18.0 (4.7)	23.1 (3.9)	8.1 (5.6) E	22
Apatemon gracilis	9	15	40.4 (18.1)	19.7 (20.2)	62.1 (29.7)	63.5 (25.5)	90
Microsomacanthus sp. T	5	58	43.2 (31.8)	27.8 (17.2)	57.4 (24.8)	29.6 (17.8)	92
*Dubininolepis podicipina**	29	65	43.8 (10.9)	30.3 (14.0)	61.8 (14.9)	31.5 (17.5)	92
Schistocephalus solidus	3	2	47.1 (8.9)	45.4 (7.5)	49.2 (10.4)	5.6 (0.9)	20
Contracaecum ovale	11	4	47.5 (20.5)	38.5 (24.5)	55.5 (22.7)	27.4 (25.3) E	81
Diorchis sp. P*	26	1,635	51.2 (4.5)	34.0 (7.7)	77.2 (10.9)	43.2 (15.0) E	82
*Tatria decacantha**	26	53	52.6 (7.5) P	45.5 (7.0)	67.0 (13.9)	21.7 (12.8)	62
Lateriporus skrjabini	4	295	52.8 (20.2)	47.8 (23.4)	63.2 (16.3)	20.6 (7.9)	51
Echinatrium sp. Y	2	19	55.1 (3.6)	48.9 (2.0)	75.6 (11.5)	26.7 (9.5)	36
Ligula intestinalis	1	4	60.0	37.5	72.5	35.0	35
Microsomacanthus sp. W	1	12	62.5	56.3	83.8	27.5	28
Parafimbriaria websteri	2	3	70.0 (3.5)	66.9 (8.0)	73.1 (0.9)	12.5	13
Capillaria obsignata	12	5	72.2 (25.7)	61.7 (29.7)	77.0 (27.3)	27.5 (25.3)	91
*Wardium paraporale**	31	1,183	74.4 (13.7)	50.4 (13.1)	94.7 (8.2)	43.9 (13.6)	70
Polymorphus marilis	1	10	81.0	66.3	87.5	21.5	22
Microsomacanthus sp. Z	3	19	92.7 (3.3)	83.4 (8.4)	97.6 (2.7)	14.1 (10.0)	28

* Core species (see text).

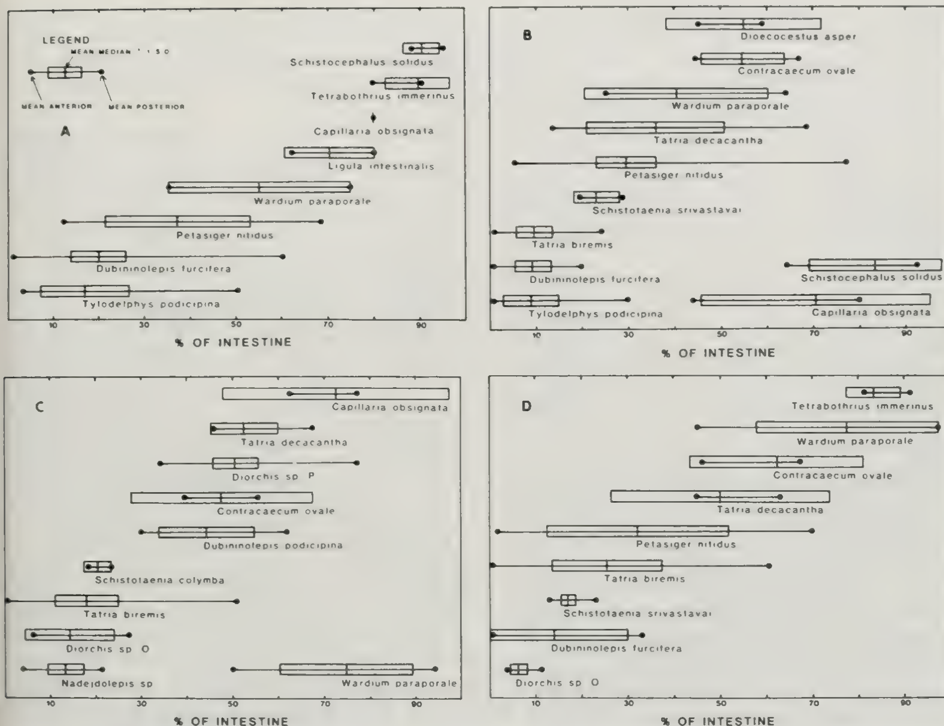

FIGURE 4. Distributions of the most common helminths in the intestines of 4 grebes. A = *Aechmophorus occidentalis,* B = *Podiceps grisegena,* C = *P. nigricollis,* D = *P. auritus.*

host are presented in Figure 4. Note that in each grebe, these helminth species are spread, although not too evenly, throughout the intestine.

To determine if the order of occurrence of these helminths in each host is predictable, all helminth species were ranked by order of position in each host, and Spearman rank correlations were calculated for all pairs of infracommunities sharing 5 or more helminth species. Although rho can vary from -1 (a negative correlation) to $+1$, the 936 comparisons were concentrated near $+1$ (85% > 0.7). Mean values of rho ranged from a low of 0.714 for *A. occidentalis,* to 0.851 for *P. auritus* (Table VI). The order of occurrence of core and recurrent group helminths along the intestinal tracts in each host species was highly predictable.

Because distributions of species may be sensitive to the size of their infrapopulations, rank correlations were calculated between the size of each infrapopulation and the position and range occupied by each helminth. The size of the infrapopulation seldom affected the location of the medianth individual; in only 6 of 51 (12%) of the cases was the location significantly ($P < 0.05$) related to population size (data in Tables II–V). Five of these 6 moved posteriad (i.e., towards the ileocaecal junction) in conjunction with increasing populations. *Schistotaenia srivastavai* in *P. auritus* was unusual in that its range was significantly reduced in larger infrapopulations; in addition, its medianth individual moved anteriad. The ranges of helminths in the intestines of grebes were more sensitive to infrapopulation size. There was a significant expansion for 20 of 48 (42%) of the species (Tables II–V), whereas 94% had positive correlations. Although the order of occurrence and positions of helminths were predictable and unresponsive to increasing infrapopulation sizes, the ranges of the parasites generally expanded when infrapopulations increased in size.

Total occupied ranges for core species (Fig. 5; Tables II–V) were generally over 60% of the intestinal length (21 of 32 cases), with 14 of these over 90%. Most species (9/16) occupied over 90%

TABLE V. *Locations of intestinal helminths in 7* Podiceps auritus *from Alberta, Canada. Locations are expressed as percents of intestinal length. 0 = pylorus, 100 = ileocaecal junction, C = range contracted significantly (*P *< 0.05) with increasing populations, E = range expanded significantly (*P *< 0.05) with increasing populations, P = mean position moved posterior significantly (*P *= 0.05) with increasing populatyions, A = mean position moved anterior significantly (*P *< 0.05) with increasing populations.*

Helminth	Number infected	Mean intensity	Mean position (SD)	Mean anterior (SD)	Mean posterior (SD)	Mean range (SD)	Total occupied range
Diorchis sp. O†	3	6	6.1 (2.0)	3.9 (5.1)	10.8 (4.7)	13.9	14
Dubininolepis furcifera†	4	32	14.0 (16.3) P	0.4 (0.5)	32.9 (37.8)	32.5 (38.0) E	99
Schistotaenia colymba	1	2	15.0	12.5	17.5	5.0	5
Schistotaenia srivastavai†	3	6	17.0 (1.1) A	13.2 (3.0)	23.0 (5.2)	13.1 (3.3) C	17
*Tatria biremis**	7	1,616	26.2 (12.2)	0.4 (0.9)	61.4 (21.2)	61.0 (21.1)	89
Petasiger nitidus†	3	131	32.2 (20.1)	1.5 (1.0)	69.5 (35.1)	68.0 (36.0)	94
Dubininolepis podicipina	1	5	38.8	32.5	43.8	11.3	11
Tatria decacantha	2	13	50.1 (23.9)	45.5 (21.5)	63.5 (34.3)	17.9 (12.8)	67
Diorchis sp. P	1	23	53.6	43.6	70.0	26.5	26
Ligula intestinalis	1	1	57.5	57.5	57.5	—	—
Contracaecum ovale†	4	4	62.6 (18.4)	46.3 (27.8)	66.8 (20.0)	27.4 (25.9) E	65
*Wardium paraporale**	7	210	77.9 (19.1)	45.3 (23.1)	97.7 (3.3)	52.4 (23.1)	82
Tetrabothrius immerinus	3	2	83.8 (8.8)	81.7 (9.0)	91.3 (2.2)	9.6 (10.2)	21
Apatemon gracilis	1	1	87.5	87.5	87.5	—	—

* Core species (see text).
† Secondary species (see text).

of the intestine in at least 1 host species. If this measure is considered the best estimate of the fundamental niche, most core species had fundamental niches that encompassed almost the entire small intestine. They showed great adaptability, indicative of broad tolerance. Only a few species of cestodes, notably the species of *Schistotaenia,* that had maximum ranges equal to or less than one-third of the length of the intestine, appeared to be limited by their tolerance to intestinal conditions.

Realized niches were considerably smaller. Mean ranges were 17–76% (mean: 46%) of the total occupied ranges (Fig. 5). From the previous analysis, 1 factor leading to these reductions is small infrapopulations. However, if reductions were due solely to small infrapopulations, then little variance about the mean of the median positions, and little variance in the mean median positions of helminths in different grebe species, would be expected. Neither was true. Although half of the core species showed small standard deviations (<10% of the intestinal length), 10 of the cases showed standard deviations of more than 15%. In addition, the positions of 9 of the 13 core species that occurred in 2 or more species of hosts varied significantly in position among host species (Table VII; Fig. 6). These analyses suggest that although the intestinal helminths in grebes are potentially capable of inhabiting large areas within the intestine, they actually reside in smaller, more localized regions that may differ among different host species, or among individuals of the same host species. These reductions can not be attributed solely to reduced infrapopulations.

When helminths were core species in both *P. grisegena* and other grebes, they had more anteriad distributions in *P. grisegena*: *D. furcifera, Tylodelphys podicipina, Petasiger nitidus,* and *Wardium paraporale* were more anteriad when in *P. grisegena* than in *A. occidentalis*; *W. paraporale, Tatria biremis,* and *Tatria decacantha* were more anteriad in *P. grisegena* than in *P. nigricollis* or *P. auritus* (Fig. 6).

Consistent differences in physicochemical

TABLE VI. *Spearman rank correlations of positions of helminths in the intestines of 4 species of grebes.*

	Aechmophorus occidentalis	*Podiceps grisegena*	*Podiceps nigricollis*	*Podiceps auritus*
No. of comparisons	53	490	388	5
No. of significant comparisons ($P \leq 0.05$)	17	319	310	3
% Significant	32	65	80	60
Mean rho	0.714	0.756	0.839	0.851
(SD)	(0.234)	(0.183)	(0.193)	(0.123)

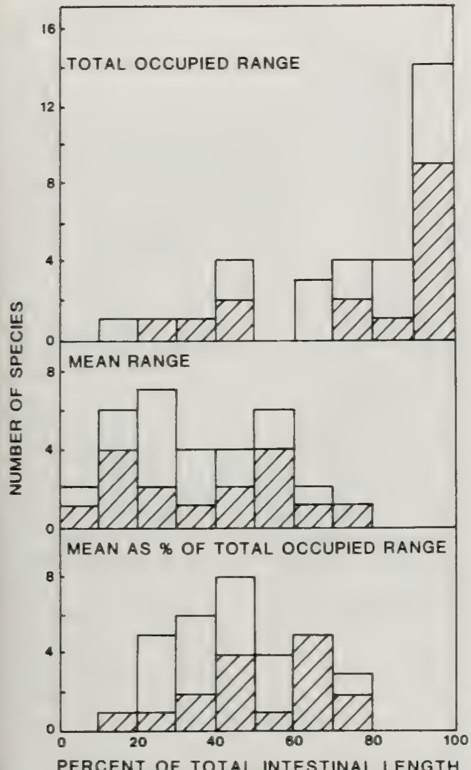

FIGURE 5. Frequency distributions of core helminths based on their total occupied ranges, mean ranges, and the mean as a percentage of the total occupied range. (Cross-hatching indicates the maximum in any of the 4 hosts.)

conditions in the luminal and mucosal environments among grebes (particularly between *P. grisegena* and the other grebes) could account for such a pattern. However, when helminths occurred as secondary or satellite species in grebes other than *P. grisegena,* they occasionally were more anteriad in position in those grebes. For example, *Schistotaenia srivastavai* was more anteriad in both *P. nigricollis* and *P. auritus,* and *Tylodelphys podicipina* and *Petasiger nitidus* were both more anteriad in *P. nigricollis.* In addition, if one assumes that the position of a species in the major or required host (that host that maintains the bulk of the parasite's breeding population) is optimal for that helminth, then examination of its distribution in other hosts should reveal consistent anteriad or posteriad shifts if physicochemical conditions vary uniformly. No such consistent shifts were noted. These observations suggest that there were no uniform physicochemical differences between the intestines of *P. grisegena* and the other hosts that might account for the consistent anterior positioning of shared core species, and that some other factor must be involved.

TABLE VII. *Summary of 1-way ANOVA's comparing distributions of medians between hosts to those within hosts.*

Helminth	Degrees of freedom	*F*-statistic	Probability
Dubininolepis furcifera	3.58	9.975	0.0000
Petasiger nitidus	3.52	2.837	0.0469
Wardium paraporale	3.84	20.853	0.0000
Capillaria obsignata	3.49	0.851	0.4727
Tylodelphys podicipina	2.44	5.099	0.0102
Tatria biremis	3.69	13.377	0.0000
Ligula intestinalis	3.24	0.293	0.8301
Contracaecum ovale	3.46	4.307	0.0093
Tatria decacantha	3.53	7.524	0.0003
Diorchis sp. P	2.25	9.538	0.0008
Dubininolepis podicipina	2.28	0.798	0.4603
Schistotaenia srivastavai	2.27	4.343	0.0232
Diorchis sp. O	2.27	0.603	0.5542

We have 3 lines of evidence that interactions among helminth species are involved. First, congeneric helminths had complementary distributions. Five sets of congeners, all cestodes, occurred in these grebes: *Tatria biremis* and *T. decacantha* co-occurred in all 4 host species, *Dubininolepis furcifera* and *D. podicipina* co-occurred in all but *P. grisegena, Diorchis* sp. O and *Diorchis* sp. P and *Schistotaenia colymba* and *S. srivastavai* co-occurred in *P. nigricollis* and *P. auritus,* and *S. srivastavai* co-occurred with a single individual of *S. tenuicirrus* in *P. grisegena.* In 6 of 12 cases, the congeners demonstrated largely disjunct distributions (average ranges, as indicated by mean posterior position of the anterior species and the mean anterior position of the posterior species do not overlap) (Tables II–V). In 3 others, ranges overlap, but mean positions are in nonoverlapping portions of the ranges. Two (the 2 *Tatria* spp. in *P. auritus* and the 2 *Dubininolepis* spp. in *A. occidentalis*) had broadly overlapping ranges but widely separated mean positions. Only *S. colymba* and *S. srivastavai* in *P. auritus* had essentially the same ranges and mean positions; they did not co-occur in the same host individual. The distributions of these 2 species were particularly interesting. *Schistotaenia srivastavai* occurred predominantly (94% of all individuals) in *P. grisegena* whereas 98% of *S. colymba* infected *P. nigricollis.* In their usu-

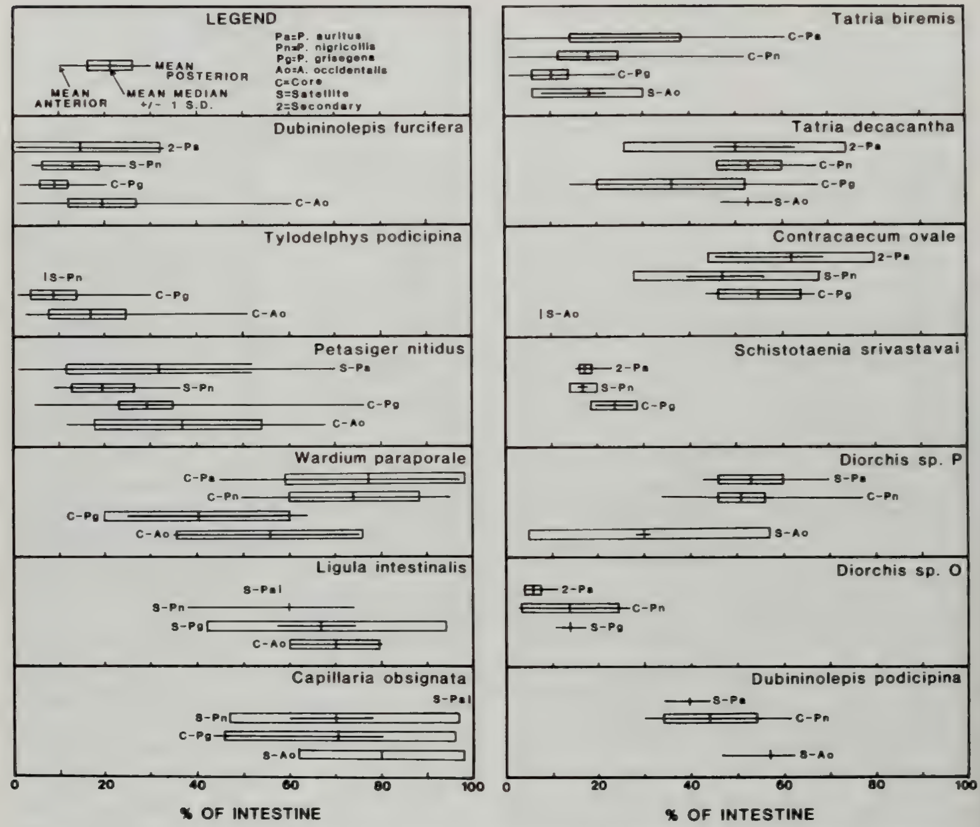

FIGURE 6. Distributions and status (core, secondary, or satellite) of helminths that occurred in more than 1 host species.

al hosts, the 2 species occupied essentially the same areas. However, when *S. srivastavai* occurred in *P. nigricollis*, its position was displaced anteriorly. Both *S. colymba* and *S. srivastavai* occurred more forward in *P. auritus* than in their main hosts (15% vs. 23% for *S. srivastavai*; 17% vs. 21% for *S. colymba*). Although these events occurred too rarely to be analyzed statisically, the median position of either species in grebes other than its main host lay outside the standard deviation of the median position in its main host. The 2 species of *Schistotaenia* differed primarily in host specificity, but had similar distributions in their main hosts; in other hosts, each assumed a more anterior position.

The remaining sets of congeners usually cooccur. Two sets, *Dubininolepis* spp. and *Diorchis* spp., regularly co-occurred only in *P. nigricollis* (see Stock and Holmes, 1987a), whereas *Tatria biremis* and *T. decacantha* regularly co-occurred in both *P. grisegena* and *P. nigricollis*. All demonstrated complementarity of distribution with little overlap, as measured by percent similarity (15% for *T. biremis* and *T. decacantha* in *P. grisegena* and 3% in *P. nigricollis*; 7% for *D. furcifera* and *D. podicipina*; and 0.4% for *Diorchis* sp. O and sp. P). The species of *Tatria* also demonstrated complementarity of numbers in *P. grisegena* and *P. nigricollis*. In *P. grisegena*, *T. biremis* had a mean intensity of 377 and *T. decacantha* a mean intensity of 1,085; in *P. nigricollis*, however, *T. biremis* had a mean intensity of 2,794 and *T. decacantha*, 53. In *P. grisegena*, its main host, increasing numbers of *T. decacantha* were correlated with a significant reduction in the ranges of the more anteriorly positioned *T. biremis* ($r = -0.392$, df $= 30$, $0.01 < P < 0.05$). This effect was not seen in *P. nigri-*

collis. In *P. nigricollis*, *Diorchis* sp. P was found in the midintestine (mean median = 51%). Overlap with its congener, *Diorchis* sp. O, was small, 0.4%, and no correlations between them indicative of interaction were noted. *Diorchis* sp. P did have a significant effect on 2 other species. Increasing numbers of *Diorchis* sp. P were associated with posterior shifts in the anteriormost and median individuals of *Wardium paraporale* ($r = 0.360$, df = 29, $P < 0.05$; and $r = 0.324$, df = 29, $0.05 < P < 0.10$, respectively) and *T. decacantha* ($r = 0.569$, df = 21, $P < 0.01$; and $r = 0.783$, df = 21, $P < 0.001$, respectively). *Podiceps nigricollis* is not the main host for either *W. paraporale* or *T. decacantha*, and both species normally occur posterior to *Diorchis* sp. P. No signs of interaction were noted between the species of *Dubininolepis* in *P. nigricollis*, and overlap was small. Therefore, congeners specialized in habitat use in different ways. *Schistotaenia* species were host species specialists, *Diorchis* and *Dubininolepis* species were microhabitat specialists, and *Tatria* species combined microhabitat separation with concentration in different host species.

Second, as demonstrated by *Diorchis* sp. P, distributional patterns of species other than congeners can indicate interactions. One method of scanning for such interactions is to look for significant reductions in the overlaps between the realized niches, compared with the overlaps between the fundamental niches, of pairs of species. No reduction is suggestive of no interaction. Overlaps in the fundamental niches of all pairs of core species were calculated using percent similarity of distributions (Bush, 1980; Bush and Holmes, 1986); overlaps in realized niches in each bird were calculated in the same way. For each pair of species, the overlap in fundamental niches was compared with the average overlap in realized niches by a t-test. Species pairs with fundamental niches that overlapped less than 5% were disregarded. Overlaps in realized niches were significantly reduced ($P < 0.05$) in many of the species pairs (35/36 in *P. grisegena*, 6/7 in *P. nigricollis*, and 5/10 in *A. occidentalis*; but only 2/7 in *P. auritus*, in which sample sizes were very small) (Fig. 7).

Almost all helminth species had expanded ranges when their population sizes were large. Therefore, the overlap between any 2 species would be expected to increase when joint population sizes of the 2 were high, unless interactions reduced overlap; this provides a second method for scanning for interactions. However, overlap between pairs of recurrent group species seldom showed a significant correlation with their combined population densities (Fig. 8). In 4 of 7 cases in which there was a significant correlation (those involving *Petasiger nitidus* or *Capillaria obsignata*), the species use intestinal resources differently. *Petasiger nitidus* is a trematode that engulfs host tissue and is closely associated with the mucosal surface; in *P. grisegena* it had increased overlap with 3 cestodes, *Dubininolepis furcifera*, *Tatria decacantha*, and *Schistotaenia srivastavai*, which absorb nutrients from the luminal contents. *Petasiger nitidus* did not have increased overlap with the other common trematode, *Tylodelphys podicipina*. Instead, increasing numbers of *P. nitidus* were correlated with a forward movement of *T. podicipina*, as shown by correlations with both the positions of posterior ($r = -0.398$, df = 26, $P < 0.05$) and medianth ($r = -0.324$, df = 26, $0.05 < P < 0.10$) individuals.

Other significant increases in overlap associated with increasing populations of helminths in *P. grisegena* were between a blood- and tissue-feeding nematode, *Capillaria obsignata*, and a lumen-dwelling cestode, *Wardium paraporale*, and between the small, paramucosal *Tatia biremis* and the large, lumen-dwelling *S. srivastavai*.

In *P. nigricollis*, no significant increases in overlap were seen. In *P. auritus*, *T. biremis* and *W. paraporale* had a significant increase. In *A. occidentalis*, however, the only case of increasing overlap was between 2 large, lumen-dwelling absorbers, *W. paraporale* and *Ligula intestinalis*. Both are residents in the posterior third of the intestine. *Aechmophorus occidentalis* is the main host for both, but *W. paraporale* is a grebe specialist whereas *L. intestinalis* is a transient generalist with a short life span in the definitive host.

Third, we have shown elsewhere (Stock and Holmes, 1987b) that the presence of one of the core species of *P. grisegena*, *Dioecocestus asper*, was associated with spatial displacement of other core species.

DISCUSSION

The intestinal helminth communities of grebes were generally large, species rich, and contained significant proportions of co-occurring species (Stock and Holmes, 1987a); all are characteristics of (and prerequisites for) interactive communities. In general, the patterns of resource exploi-

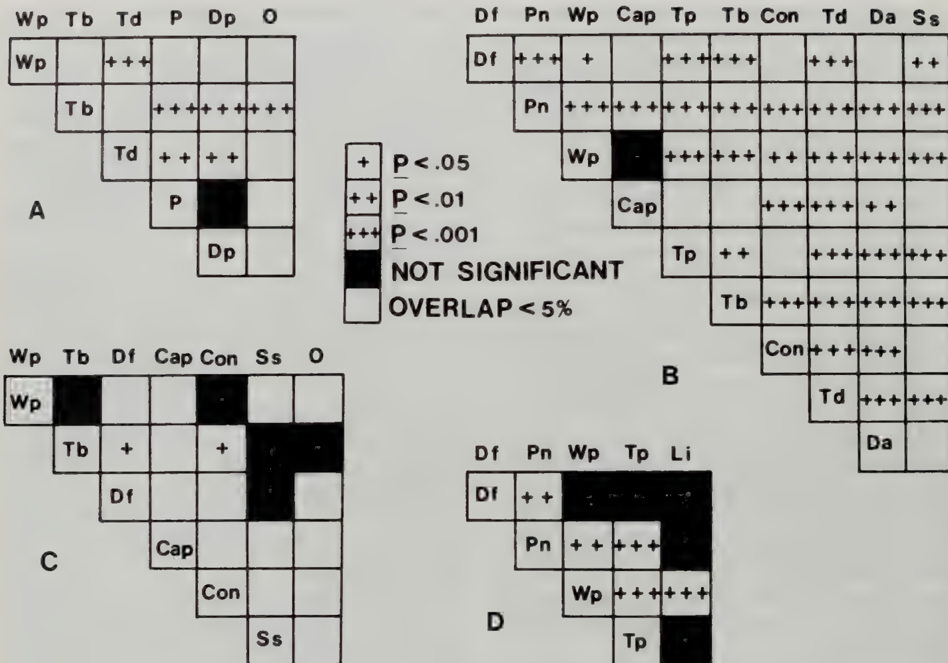

FIGURE 7. Reductions in overlaps of realized niches of core helminths in 4 grebes. A = *Podiceps nigricollis*, B = *P. grisegena*, C = *P. auritus*, D = *Aechmophorus occidentalis*. Cap = *Capillaria obsignata*, Con = *Contracaecum ovale*, Da = *Dioecocestus asper*, Df = *Dubininolepis furcifera*, Dp = *Dubininolepis podicipina*, Li = *Ligula intestinalis*, O = *Diorchis* sp. O, P = *Diorchis* sp. P, Pn = *Petasiger nitidus*, Ss = *Schistotaenia srivastavai*, Tb = *Tatria biremis*, Td = *Tatria decacantha*, Tp = *Tylodelphys podicipina*, Wp = *Wardium paraporale*.

tation, based on microhabitat distributions, supported the conclusion that these are interactive communities.

The fundamental niches of the common parasites in the intestines of grebes were generally broad, encompassing most of the intestine. Their realized niches, however, were small subsets of their fundamental niches, and when parasites occurred in more than 1 host species, they frequently occupied different positions. Nevertheless, the order of occurrence in relation to the other common species in each infracommunity was predictable. These features suggest that the degree of specialization of helminths to specific parts of the intestine does not limit them to a precise site, as assumed for isolationist communities; however, specialization may determine where they do best.

Bush and Holmes (1986) pointed out several caveats that must be considered in any study using similar techniques and analyses. Drawing interpretations from linear distributions alone, we assume that niche parameters important to each helminth species are indicated by their position in the intestine. Although the linear axis is likely to be very important, the radial, circumferential, or resource-use axes may also be significant. Also, helminths may migrate, or due to senescence, may be involuntarily removed from their usual location, leading to the false impression of wide fundamental niches. Although few distributional gaps, posteriorly skewed distributions, or other indications of senescent worms were noted, we cannot reject the possibility that this occurs in some cases.

Several analyses provided direct evidence for interactive communities. Realized niches of adjacent species were arranged so as to markedly reduce the potential overlaps indicated by their fundamental niches. Overlaps between species seldom increased when joint population size increased. Congeners that regularly co-occurred in the same infracommunities occupied complementary ranges. Finally, the distributions and abundances of some helminths in grebes, especially *Dioecocestus asper* and *Diorchis* sp. P, did

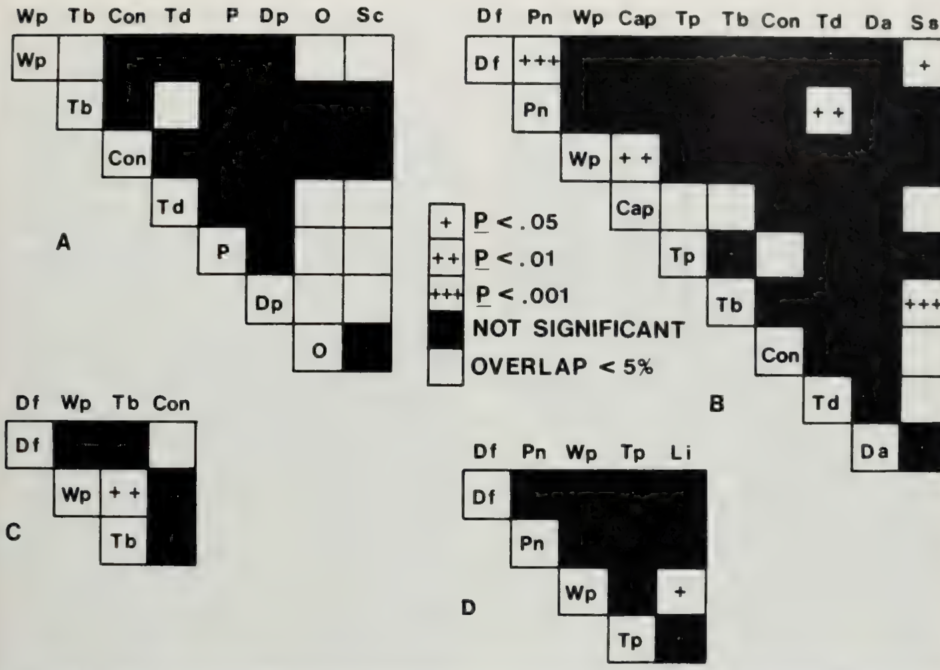

FIGURE 8. Significant increases in overlap between intestinal helminths with increasing combined populations in 4 grebes. A = *Podiceps nigricollis*, B = *P. grisegena*, C = *P. auritus*, D = *Aechmophorus occidentalis*. Helminth species initials as for Figure 7, plus Sco = *Schistotaenia colymba*.

affect those of other species. *Dioecocestus*, or the pathological changes to the mucosal surface of *P. grisegena* that it induces, affected the distributions within the intestine of other core species and eliminated satellite species from infracommunities. Stock and Holmes (1987b) concluded that it was acting as an interference competitor. Therefore, several lines of evidence indicate that interactions between helminths are important in determining the structure of infracommunities in grebes.

However, 2 of the analyses did not support the conclusion of interactive communities. The presence of many open sites (vacant niches) is generally considered characteristic of isolationist communities (Rohde, 1977, 1979; Price, 1984). Infracommunities in each of the grebe species included vacant sections, particularly toward the posterior end of the intestine. Vacant niches may be even more common. Intestinal parasites may be allocated to 3 different guilds on the basis of how and where they acquire nutrients (Bush, 1980; Bush and Holmes, 1986). Trematodes and nematodes (the engulfer guild) have intestinal tracts and typically engulf host tissues, sloughed epithelial material, and luminal contents. Cestodes and acanthocephalans absorb nutrients through their body surfaces. Small absorbers are generally intimately associated with the host's mucosal surface, the paramucosal zone, whereas larger absorbers frequently attach their scolices to the mucosal surface, but retain the bulk of their biomass in the lumen. Figure 9 shows the combined average distributions of the core species belonging to each of the 3 guilds in each host species. It is apparent that all grebes lack large, lumen-dwelling absorbers in the anterior intestine and small, paramucosal-dwelling absorbers and engulfers in the posterior intestine. The helminths of grebes do not appear to form saturated communities; vacant niches, indicative of isolationist communities, occur in most infracommunities and in the overall component communities.

In addition, in interactive communities, core species should be spread more evenly in niche space than would be expected by chance alone (Hanski, 1982; Bush and Holmes, 1986). However, broken-stick analyses indicated that none of the grebes had communities in which core

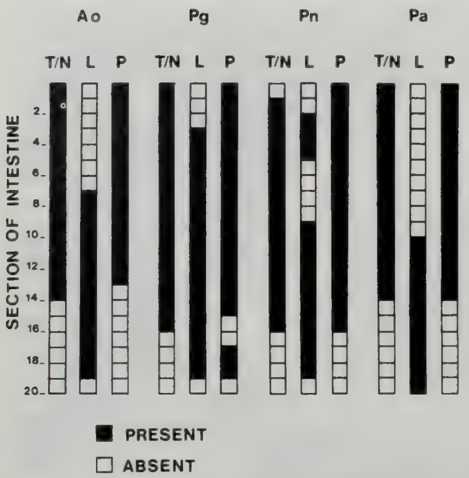

FIGURE 9. The distribution of 3 guilds in the intestines of grebes. T/N = trematodes and nematodes (the engulfer guild), L = lumen dwellers, P = paramucosal dwellers. Ao = *Aechmophorus occidentalis*, Pg = *Podiceps grisegena*, Pn = *P. nigricollis*, and Pa = *P. auritus*.

species were spread significantly more evenly. This again suggests the presence of isolationist communities; however, it may also imply that the entire intestine is not equally available or suitable for occupation (the posterior terminal distances in *P. grisegena* infracommunities were frequently the largest).

Patterns of microhabitat distribution of helminths in lesser scaup, *Aythya affinis* (Bush and Holmes, 1986), are directly comparable to those observed in grebes. Bush and Holmes found that the fundamental niches of core species were large (over half the intestine) whereas realized niches were much smaller. Overlaps between species did not increase with increasing joint population size. Unlike the situation in grebes, core species in scaup were distributed more evenly than could be expected by chance alone. However, as in grebes, the community was not saturated (the trematode/nematode engulfer guild was frequently absent). Nevertheless, the core and secondary absorber species in scaup provided an interactive structure to the community. Therefore, the patterns of distribution of helminths in both scaup and grebes indicate that empty niches do not necessarily provide evidence for isolationist communities.

Butterworth (1982) identified a cline in the structure of communities of helminths in waterfowl ranging from those that were small, simple, and unpredictable (isolationist communities, e.g., those in widgeon, *Anas americana*) to those that were large, complex, and predictable (interactive communities, e.g., those in white-winged scoter, *Melanitta fusca*, or in lesser scaup). The communities in grebes were much more predictable than waterfowl communities with similar sizes and complexities (see Stock and Holmes, 1987a). Although interactions among helminths in grebes are important, they are not as pervasive as in the scaup system (Bush, 1980), possibly because of the smaller, simpler communities in grebes. Analysis of data presented in Pojmanska (1982) showed that interactions among 3 species of *Diorchis* in coots (*Fulica atra*) regulated the spatial distributions of these tapeworms only when infrapopulations were high. When populations were low, the communities resembled isolationist assemblages.

Holmes and Price (1985) predicted the occurrence of 2 types of parasite communities: assemblages of microhabitat specialists, with little or no interactions occurring in nonequilibrium, unsaturated communities; and assemblages of species with high density populations in equilibrium communities in which interactions are significant. However, this simplistic view is inadequate. Interactive and isolationist communities more accurately represent the ends of a continuum with communities such as those in lesser scaup close to one end, and those in hosts like widgeon close to the other. Enteric communities of grebes were intermediate and affected by both sets of processes. Grebes have unsaturated communities in which interactions do occur, but are less significant than those noted in scaup (Bush, 1980; Bush and Holmes, 1986). If important resources for some guilds are not distributed evenly along the enteric gradient, then interspecific interactions may be significant only in some regions of the habitat, and specializations may be more crucial in other areas. This possibility will be explored in a separate paper.

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PATHOGENICITY OF *HAEMOPROTEUS MELEAGRIDIS* (HAEMOSPORINA: HAEMOPROTEIDAE) IN EXPERIMENTALLY INFECTED DOMESTIC TURKEYS

Carter T. Atkinson*, Donald J. Forrester, and Ellis C. Greiner

Department of Infectious Diseases, College of Veterinary Medicine, Box J-137, University of Florida, Gainesville, Florida 32610

ABSTRACT: Sporozoite-induced experimental infections of *Haemoproteus meleagridis* produced a moderate to severe myositis and significant effects on weight gain and growth in domestic turkey poults. Pathological effects occurred in both low- and high-dose infections (4,400 and 57,500 sporozoites, respectively). Low-dose birds weighed significantly less than controls at 3 wk postinfection (PI) when peripheral parasitemia reached a peak and had significantly shorter tarsometatarsal lengths at both 1 and 3 wk PI. High-dose birds were significantly lighter and smaller than control and low-dose birds throughout the course of the 8-wk study. Infected birds were not anemic in spite of high parasitemias that often exceeded 50% of circulating erythrocytes. The most serious pathological effects occurred prior to patency and were associated with development of megaloschizonts in skeletal muscle. Microscopic lesions in 4 high-dose birds that died between 19 and 22 days PI were characteristic of a severe, acute hemorrhagic myositis. Megaloschizonts were surrounded by a hemorrhagic inflammatory infiltrate composed of macrophages, heterophils, giant cells, and red blood cells. Muscle fibers adjacent to megaloschizonts were swollen, hyaline, and contained prominent calcium deposits. Other observations included enlargement of the spleen, deposition of pigment in macrophages of the lung and spleen, and secondary bacterial and fungal infections in the intestine and lungs. Necrotic and calcified muscle fibers and degenerating megaloschizonts were still present at 8 wk PI when the experiment ended. Our results demonstrated significant pathological changes in *H. meleagridis*-infected domestic turkeys that were associated primarily with preerythrocytic stages of development.

Since the first detailed studies of *Haemoproteus* spp. in the early part of this century, a number of authors have reported instances where avian haemoproteids appeared to be pathogenic. Acton and Knowles (1914), Adie (1924), Coatney (1933), and Markus and Oosthuizen (1972) observed weakened, anemic, and anorexic pigeons with intense infections of *H. columbae.* Becker et al. (1956) attributed enlargement and discoloration of spleens in pigeon squabs to infection by *H. sacharovi.* In an extensive study of *H. lophortyx* in naturally infected California quail, O'Roke (1930) described 4 stages of disease ranging from a mild-chronic condition with no obvious signs of infection to rare severe-acute infections where birds were weakened, anorexic, and eventually died. More recently, inflammatory reactions and an associated myopathy have been described around megaloschizonts of *H. handai* (=*H. desseri*) and *H. meleagridis* in parakeets and turkeys, respectively (Miltgen et al., 1981; Atkinson et al., 1986; Atkinson and Forrester, 1987). Some workers have speculated that *H. columbae* and other avian haemoproteids must be pathogenic to some extent because peak parasitemias may involve half of the circulating erythrocytes (Garnham, 1966; Levine, 1973). Nevertheless, the most widely held view is that these organisms are relatively benign parasites that are well adapted to their hosts.

In spite of numerous empirical reports, there have not been any attempts to assess the pathogenicity of avian haemoproteids in a suitable experimental model. In this study we used sporozoite-induced experimental infections to determine the effects of *H. meleagridis* from wild turkeys on growth, weight gain, and selected hematologic parameters in 1-wk-old domestic turkey poults.

MATERIALS AND METHODS

Thirty-six, 1-day-old, female, broad-breasted white turkey poults were obtained from Thaxton's Turkeys (Watkinsville, Georgia). They were housed together in a brooder in a vector-proof room for 7 days, then banded with metal wing tags and randomly assigned to 3 experimental groups. Birds in the first group were inoculated intraperitoneally (IP) with separate pools of 5 *Culicoides edeni* that had taken blood meals from domestic poults infected with *H. meleagridis* 8–9 days earlier as described by Atkinson et al. (1986). Briefly, 4 2-wk-old domestic turkeys were exposed in a sentinel cage in wild turkey habitat at Lyke's Fisheating Creek Wildlife Refuge (Glades County, Florida) for 1 wk and held in vector-proof isolation facilities as described by

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* Present address and address for reprints: Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106.

Atkinson et al. (1988). Previous surveys had demonstrated that *H. meleagridis* had a prevalence of almost 90% in wild turkeys from this area (Forrester et al., 1974). Poults that developed patent infections of *H. meleagridis* were exposed in a Bennett trap in the forest canopy on 2 consecutive evenings at Lyke's Fisheating Creek Refuge for collection of *Culicoides* vectors. Engorged *Culicoides* were aspirated from the trap and held for 7 days at room temperature in screened cardboard cartons as described by Atkinson et al. (1983) to allow development of sporozoites. Gravid *Culicoides* were aspirated from the cartons, identified by wing pattern, and held in RPMI-1640 tissue culture medium on wet ice. Pools of *C. edeni* were ground separately by hand in RPMI-1640 medium with 10% turkey serum in a Ten Broek tissue grinder held in wet ice. Each of 12 1-wk-old poults was inoculated with 0.5 ml of a slurry containing approximately 4,400 sporozoites as determined with a hemacytometer. Each of 12 poults in the second experimental group was inoculated IP as above with a pool of 35 *C. edeni* that contained approximately 57,500 sporozoites. Each of 12 control poults was inoculated IP with 0.5 ml of RPMI tissue culture fluid containing 10% turkey serum. Following inoculation, poults were randomly assigned to 12 battery cages in a vector-proof room. Birds were distributed in groups of 3 so that each battery cage compartment held a representative from each experimental group. The poults were fed a high protein unmedicated game bird feed (Purina Game Bird Chow) and watered *ad libitum*.

Twenty-four hours prior to inoculation (week 0), and once a week for 8 wk PI, each bird was weighed and the tarsometatarsal length of the right leg was measured with calipers. Blood was collected from a wing vein into 2 heparinized capillary tubes and immediately drawn from 1 capillary tube into 2 20-μl pipettes. The contents of each pipette were then dispensed into separate test tubes containing 5 ml of cyanomethemoglobin reagent (1:251 Cyanomethemoglobin Test Kit, Fisher Scientific). The test tubes were mixed with a vortex mixer, allowed to stand for several hours, and then placed in a spectrophotometer and measured at 540 nm. Hemoglobin concentration for each paired sample was determined with a standard measured at the same time (Cyanomethemoglobin Standard, Fisher Scientific). The average value for each paired sample was used in the statistical analysis. The second heparinized capillary tube was spun for 5 min in a microhematocrit centrifuge. The packed cell volume (PCV) was determined and the plasma protein concentration was measured with a refractometer that had been calibrated with a standard bovine albumin solution (Armour Pharmaceutical Company). Blood smears were prepared from all birds 3 times per week as described by Atkinson et al. (1983). Parasitemias were determined by counting the number of gametocytes per 10,000 red blood cells.

At 4 wk PI and again at the end of the experiment, pooled fecal samples were collected from each battery cage compartment. Flotations were performed on the samples with Sheather's sugar solution to detect coccidian oocysts. At 4 wk PI, cloacal swabs were prepared from 3 to 5 randomly selected birds in each experimental group, incubated overnight in selenite enrichment media, and plated on MacConkey's agar. Bacterial colonies morphologically similar to *Salmonella* spp. were identified by biochemical reaction with Micro ID test kits (Mallincrodt Industries). *Salmonella* spp. isolates were sent to the National Veterinary Diagnostic Laboratory at Ames, Iowa for further typing. At the termination of the experiment, cloacal swabs were made from all surviving birds and screened for *Salmonella* spp. as above.

At 8 wk PI, all surviving birds were necropsied. Wet weights of heart, liver, and spleen (expressed as percent of total body weight at necropsy) were measured. Representative pieces of pectoral muscle, liver, spleen, heart, lung, brain, proventriculus, gizzard, duodenum, pancreas, ileum, jejunum, cecum, kidney, and bone marrow taken from the femur were fixed in buffered 10% formalin. The 3 lightest birds from each group were selected and all representative tissues from each were dehydrated in ethanol or isopropyl alcohol, cleared in toluene for 2 hr, embedded in paraplast, sectioned at 5 μm, and stained with hematoxylin and eosin. Representative tissues from birds that died prior to the end of the experiment were fixed in buffered 10% formalin, dehydrated, cleared, sectioned, and stained as above. Selected serial sections of skeletal muscle were stained for calcium with von Kossa's stain (Humason, 1979).

Data on weight, tarsometatarsal length, hematocrit, plasma protein concentration, and hemoglobin were analyzed with the SAS general linear models procedure as a split-plot design with subjects as main plot units and subjects at a particular time as a subplot unit (Freund and Littell, 1981). Treatment, subject(treatment), week, and treatment·week were tested for each variable using the Type III sum of squares. A P value of 0.05 or smaller was considered significant. When treatment·weeks interactions were significant, further comparisons were made by treatment and by week with Duncan's multiple range test. A comparison of organ weights at necropsy was done with a 1-way analysis of variance using the SAS general linear models procedure (SAS Institute, 1982).

RESULTS

Parasitemia

Young gametocytes appeared in the peripheral circulation of all birds in the low- and high-dose groups at day 17 PI (Fig. 1). Control birds remained uninfected. The parasitemia in both infected groups quickly reached a peak by day 21 PI. At the crisis, the low- and high-dose groups had mean parasitemias of 2,109 and 5,760 gametocytes per 10,000 red blood cells, respectively. Two birds in the high-dose group had peak parasitemias that exceeded 7,000 gametocytes per 10,000 red blood cells. Multiple infections of red blood cells were common, with some cells containing as many as 6 gametocytes. Average parasitemias in both groups rapidly fell within 7 days to values less than 10% of those at the crisis (Fig. 1). A second, smaller peak in parasitemia occurred in both groups at approximately 38 days PI. Both groups were patent throughout the study, although parasitemias were

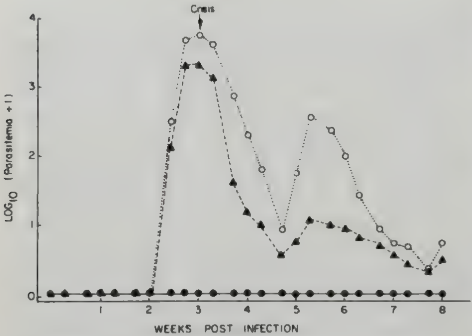


FIGURE 1. Average parasitemia for high-dose poults (O), low-dose poults (▲), and control poults (●). The crisis (arrow) occurred on the day when peak parasitemia was reached.

often less than 10 gametocytes per 10,000 red blood cells.

Gross observations

Fatal infections: As early as day 7 PI the poults in the high-dose group stood with slightly drooped wings, ruffled feathers, and partially or completely closed eyes and were lethargic when compared to birds belonging to either the low-dose or control groups. Between days 7 and 14 PI, birds in the high-dose group developed a mild diarrhea that produced a "pasty" vent. By day 15 PI, most of these birds exhibited lameness in 1 or both legs, severe depression, emaciation, dehydration, and anorexia.

Between days 19 and 22 PI, 4 birds (33%) in the high-dose group died. The deaths occurred from 2 to 5 days after the appearance of young gametocytes in the peripheral circulation. Deaths did not occur in either the low-dose or control groups. At necropsy, fusiform white cysts (1.0 mm × 0.5 mm) were scattered densely throughout the skeletal muscles of all 4 poults and were oriented parallel to the muscle bundles. From 30 to 50% of the cysts were hemorrhagic (Fig. 2). A 5-mm-diameter white nodule was observed on the cut surface of the posterior lung of 1 bird and thickened air sacs with scattered white 5-mm-diameter plaques were observed in another. In all 4 poults, portions of the duodenum, ileum, jejunum, and colon were flaccid and the mucosa had multifocal areas of reddish discoloration. Reddish or black, tarry mucoid material was occasionally present in the jejunum. A bacterial culture from 1 bird was positive for *Salmonella enteriditis* Group B.

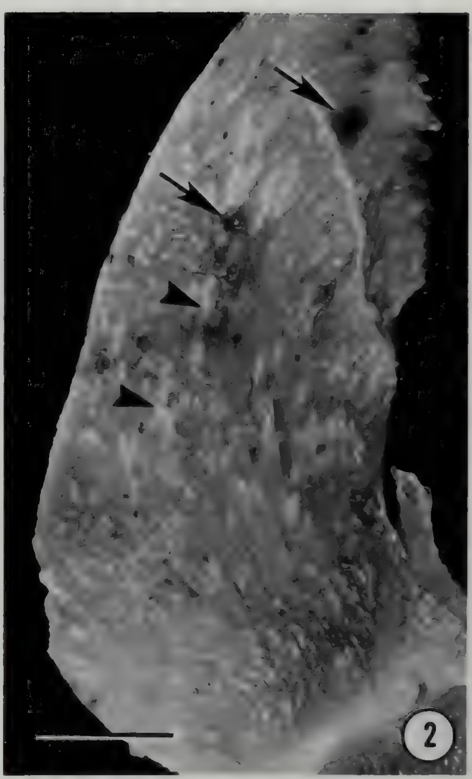
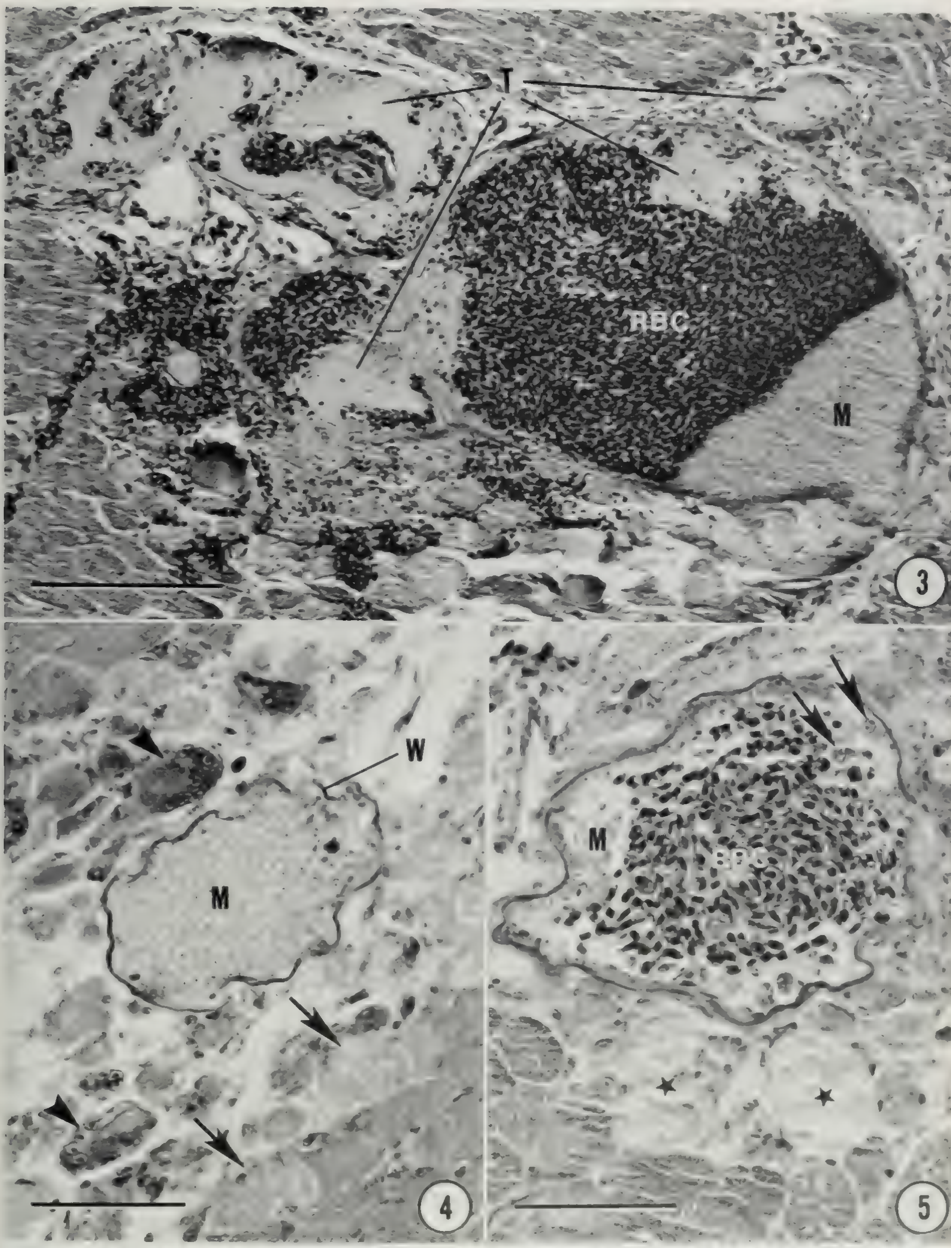


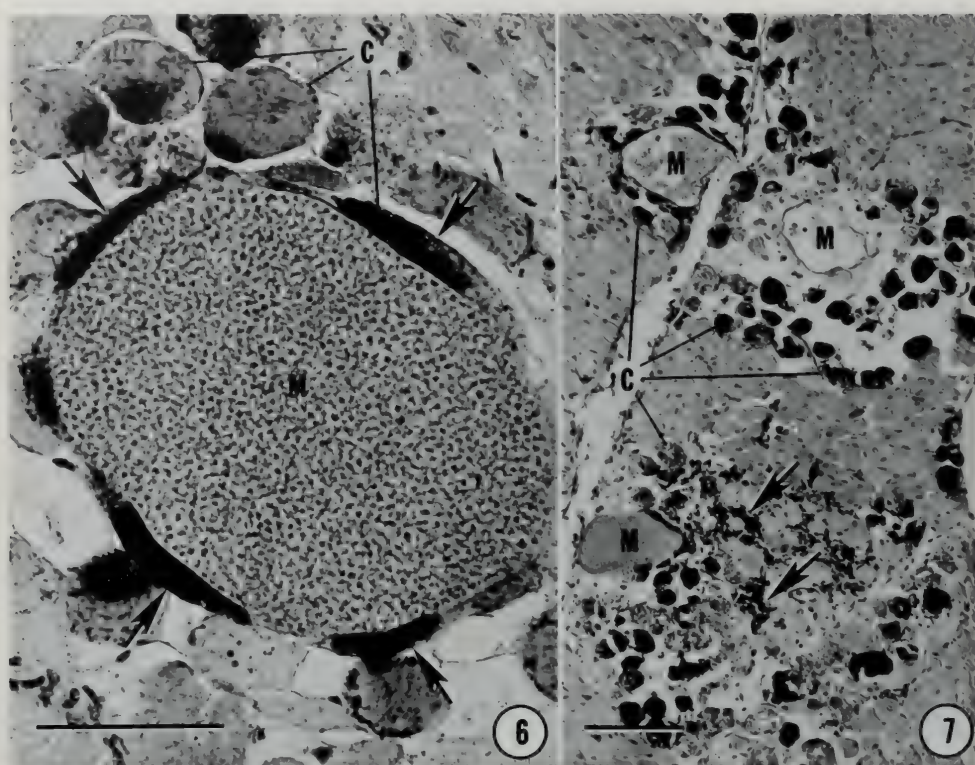
FIGURE 2. Formalin-fixed pectoral muscle from a high-dose poult that died on day 19 PI. Note the scattered white streaks (arrowheads), dark flecks, and darkened hemorrhagic areas (arrows) that corresponded to megaloschizonts in histological sections. Bar = 0.5 cm.

A randomly selected poult in the control group was killed and necropsied at day 22 PI to determine whether the muscle cysts may have resulted from contamination with *Sarcocystis.* Tissue cysts were not evident in any skeletal muscles of this bird. All organs and tissues were grossly normal. At day 27 PI, cloacal swabs from 2 of 3 randomly selected low-dose birds, 2 of 3 randomly selected control birds, and 3 of 5 randomly selected high-dose birds were positive for *Salmonella enteriditis* Group B, serotype *heidelberg.*

Surviving birds: Upon necropsy at 8 wk PI, 9 of 12 low-dose birds, and 8 of 8 high-dose birds had low numbers of fusiform, white cysts in the pectoral muscles. The cysts were 2–3 times larger and less distinct than cysts observed in the 4 fatal infections. Cysts were not detected in any of the 11 control birds. All poults had focal areas of reddened, thickened mucosa and occasional pe-



FIGURES 3–5. Ruptured and intact megaloschizonts from pectoral muscle of a high-dose poult that died on day 19 PI. H&E. **3.** Hemorrhagic megaloschizont (M) that is surrounded and partially filled by red blood cells (RBC). Thrombi (T) with embedded red blood cells are adjacent to or within the megaloschizont. Bar = 100 μm. **4.** Intact megaloschizont (M) surrounded by giant cells (arrowheads) and hyaline and necrotic muscle fibers (arrows). Note thick hyaline wall (W) surrounding the megaloschizont. Bar = 50 μm. **5.** Ruptured and hemorrhagic megaloschizont (M) that is filled with red blood cells (RBC) and macrophages (arrows). Note necrotic muscle fibers (*) adjacent to the megaloschizont. Bar = 50 μm.



FIGURES 6, 7. Megaloschizonts from pectoral muscle of a high-dose poult that died on day 22 PI. H&E. **6.** Megaloschizont (M) is surrounded by partially and completely calcified muscle fibers (C). Note distended, calcified muscle fiber (arrows) that surrounds the megaloschizont, suggesting that development of the parasite may be within the fiber. Bar = 50 μm. **7.** Lower magnification of pectoral muscle from the same poult. Three ruptured, empty, or intact megaloschizonts (M) are surrounded by darkly staining calcified muscle fibers (C). A hemorrhagic infiltrate (arrows) is adjacent to the megaloschizont in the lower half of the figure. Bar = 100 μm.

techial hemorrhages throughout the length of the intestine. None of the birds, however, exhibited clinical signs of salmonellosis, i.e., "pasty" vent, bloody diarrhea or depression. Cloacal swabs from 2 of 11 control birds, 1 of 12 low-dose birds, and 1 of 8 high-dose birds were positive for *Salmonella enteriditis* Group B. The "pasty vents" observed in high-dose birds at 1 wk PI resolved spontaneously in surviving birds by 4 wk PI. Coccidian oocysts were not detected in pooled fecal samples at either 4 or 8 wk PI. Incidental findings included the presence of numerous white nodules, approximately 5 mm in diameter, that were scattered throughout the mesentery and peritoneum of 1 control bird and 2 high-dose birds. Average wet weights of hearts and livers removed from the birds at necropsy were not significantly different for any group. Average spleen weights for all 3 experimental groups differed significantly ($P < 0.0002$) and were correlated with dose, i.e., high-dose birds had the highest average spleen weight and control birds had the lowest.

Microscopic observations

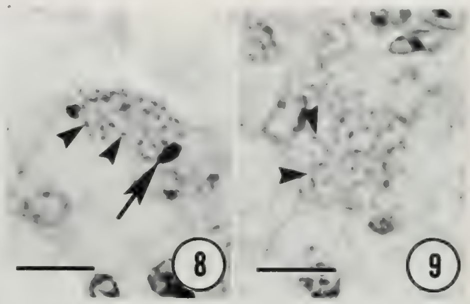
Fatal infections: The most significant microscopic lesions found in the 4 birds that died between days 19 and 22 PI were associated with numerous intact and ruptured, fusiform, intramuscular megaloschizonts. Ruptured megaloschizonts were surrounded by a hemorrhagic inflammatory infiltrate composed of macrophages, heterophils, giant cells, and red blood cells and were frequently invaded by macrophages and heterophils (Figs. 3–5). Hemorrhagic areas within and near ruptured megaloschizonts occasionally contained thrombi with embedded macrophages and heterophils (Fig. 3). Swollen, rounded,

and hyaline muscle fibers adjacent to megaloschizonts often contained small, gray to dark blue granules (Fig. 6). Dark blue calcium deposits, visible with hematoxylin and eosin as well as with von Kossa's calcium stain, often occupied much of the cytoplasm of muscle fibers adjacent to megaloschizonts (Figs. 6, 7).

Liver and spleen sections from 2 poults that died on day 22 PI, 5 days after gametocytes first appeared in circulating red cells, had numerous golden-yellow pigment deposits in the cytoplasm of macrophages. The 2 birds that died on days 19 and 20 PI lacked deposits. Spleen sections from all 4 birds had extensive areas of follicular atrophy characterized by a large reduction in the size of the periarteriolar lymphatic sheaths. Numerous mature and ruptured schizonts were present in reticular cells of the spleen of 1 bird that died on day 19 PI. These schizonts were from 10 to 15 μm in diameter and lacked the thick, hyaline wall that surrounded megaloschizonts in muscle tissue (Figs. 8, 9). Most schizonts contained small, spherical merozoites, less than 1 μm in diameter, although a few contained long slender merozoites. A section of cardiac muscle from 1 bird contained a single megaloschizont.

Incidental findings in lung tissue from 3 of the 4 birds included the presence of large granulomas composed of giant cells, mononuclear cells, heterophils, and fibroblasts that surrounded large, amorphous eosinophilic central cores which contained necrotic debris. Fungal hyphae resembling *Aspergillus* sp. were evident in lung sections from 1 bird. Endothelial cells lining alveolar capillaries were hypertrophic and associated capillaries were congested with blood cells. A fibrino-hemorrhagic exudate containing macrophages and heterophils filled alveolar spaces in focal areas of the lung. Some large blood vessels were partially or completely occluded by thrombi. Sections of air sacs of 1 bird and mesentery of another had granulomas that surrounded eosinophilic masses containing fungal hyphae. Focal areas of enteritis characterized by the presence of heterophils in the lamina propria and submucosa were present in sections of intestine and cecum. Coccidian parasites were not detected. Sections of kidney, brain, bone marrow, proventriculus, and gizzard were normal.

Surviving birds: At 8 wk PI, infiltrates of mononuclear cells, heterophils, and giant cells were evident in sections of skeletal muscle from low- and high-dose birds (Fig. 10). Necrotic and calcified muscle fibers were at the center of some



FIGURES 8, 9. Small schizonts developing in splenic reticular cells of a high-dose poult that died on day 19 PI. H&E. **8.** Host cell with pycnotic nucleus (arrow) is packed with small spherical merozoites (arrowheads), similar to those found in mature megaloschizonts. Bar = 10 μm. **9.** Reticular cell packed with long, slender merozoites (arrowheads) that are similar in morphology to merozoites from first-generation schizonts. Bar = 10 μm.

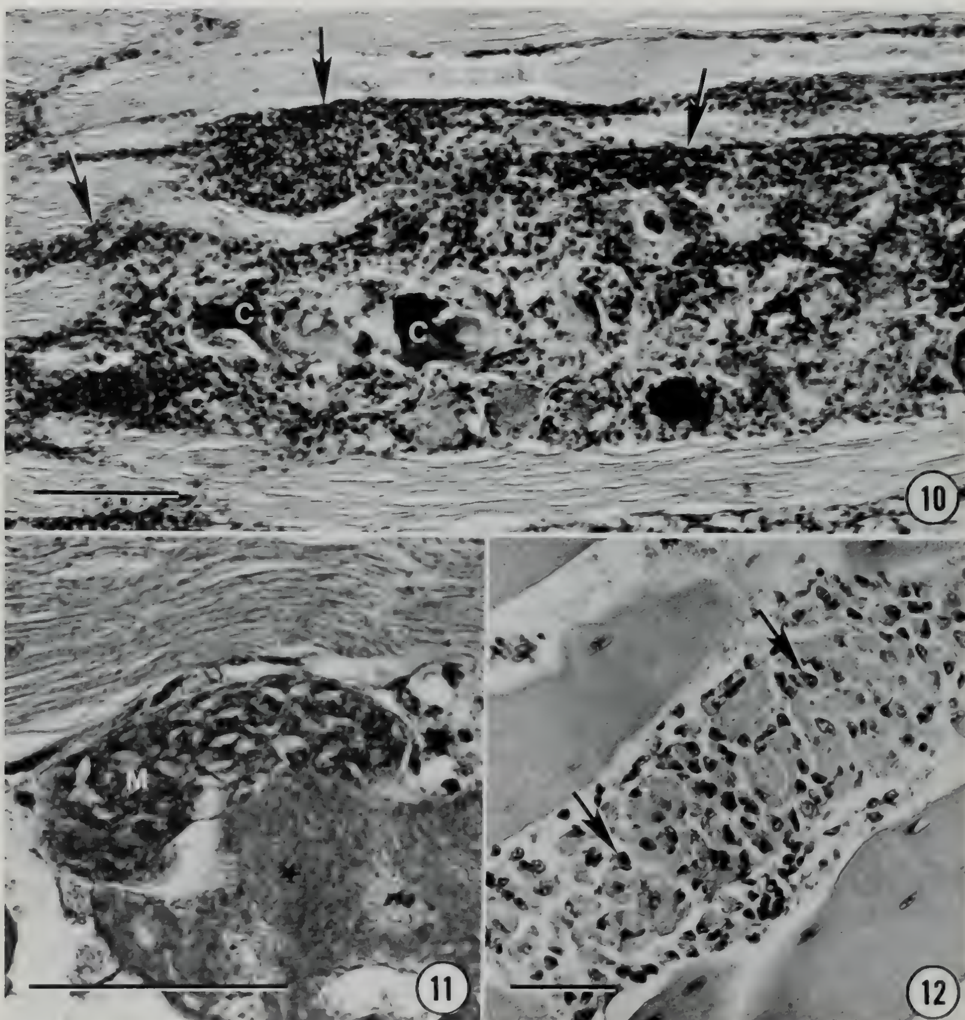
of the infiltrates. A multifocal, perivascular cuffing consisting of lymphocytes and heterophils was also present. Pectoral muscle from 1 low-dose bird contained a degenerating megaloschizont (Fig. 11). Remnants of degenerating muscle fibers, infiltrated with macrophages and heterophils, were scattered throughout the muscle tissue (Fig. 12).

Macrophages in the liver, lung, and spleen from both low- and high-dose birds contained moderate to extensive deposits of pigment. Deposits in the liver and spleen were massive and brownish-black, whereas those in the lung were smaller and golden-brown. No pigment was found in control birds. Follicular hyperplasia was common in the spleens of all infected birds. The degree of hyperplasia as well as the number of pigment deposits varied directly with the size of the infective dose.

Sections of duodenum, ileum, jejunum, and colon from the control, low-dose, and high-dose birds had a few multifocal areas of infiltrate composed of heterophils. Coccidian parasites were not observed. One high-dose bird had a granulomatous peritonitis composed of macrophages, heterophils, and giant cells that surrounded amorphous, eosinophilic masses that were scattered over the peritoneum. Sections of brain, bone marrow, kidney, heart, proventriculus, and gizzard were normal.

Weight

Statistical analysis of the weight data revealed that all 4 variables in the model statement, i.e.,



FIGURES 10–12. Lesions in pectoral muscle from poults with 8-wk-old infections of *H. meleagridis*. H&E. **10.** Infiltrate (arrows) composed of monocytes and heterophils surrounds necrotic and calcified muscle fibers (C). Bar = 100 μm. **11.** Degenerating megaloschizont (M) from a low-dose poult. A hyaline and necrotic muscle fiber (*) is adjacent to the megaloschizont. Bar = 50 μm. **12.** Necrotic muscle fiber infiltrated by monocytes and heterophils (arrows). Bar = 100 μm.

treatment, subject(treatment), week, treatment·week, were significant ($P < 0.0001$). When means were compared by week, all 3 groups had significantly different average weights at 3 wk PI during the crisis. The average weight of high-dose birds was significantly lower than that of control and low-dose birds during all other weeks. Other differences between the control and low-dose groups were not significant (Fig. 13). When means were compared by experimental group, control and low-dose birds had significant increases in average weight at each week PI. By contrast, the average weight for the high-dose group did not increase significantly between 0 and 2 wk PI, between 1 and 3 wk PI, and between 3 and 4 wk PI (Fig. 13).

Tarsometatarsal length

Statistical analysis of tarsometatarsal length revealed that all 4 variables in the model state-

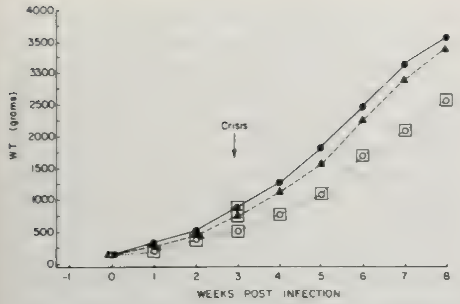


FIGURE 13. Average weights for high-dose (O), low-dose (▲), and control (●) poults with experimental infections of *H. meleagridis*. Points surrounded by a square indicate significantly different means when comparisons are made among the 3 experimental groups by week, $P < 0.05$.

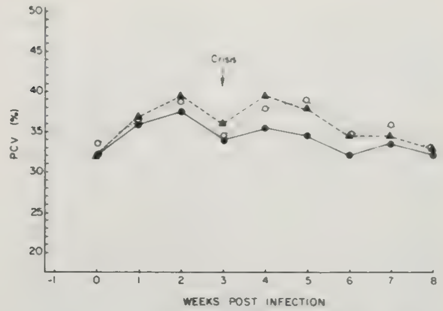


FIGURE 15. Average hematocrits for high-dose (O), low-dose (▲), and control (●) poults with experimental infections of *H. meleagridis*. Means for each experimental group were not statistically different when comparisons were made by week.

ment were significant ($P = 0.0001$). When means were compared by week, all 3 groups had significantly different average tarsometatarsal lengths at 1 wk PI and at 3 wk PI during the crisis. The high-dose group had an average tarsometatarsal length that was significantly shorter than control and low-dose birds at all other weeks PI. Other differences between the control and low-dose groups were not significant (Fig. 14). When means were compared by experimental group, each group showed a significant increase in average tarsometatarsal length for every week.

Hematocrit

Statistical analysis of hematocrit data revealed that all variables in the model statement were significant ($P = 0.0001$) except treatment·week ($P = 0.6886$). Differences in average hematocrits were not significant when averaged over all weeks ($P = 0.1617$). Differences were occasionally significant when means were compared by experimental groups, however, no consistent pattern was evident and average values for each group showed similar increases or decreases (Fig. 15).

Hemoglobin

Statistical analysis of hemoglobin data revealed that all 4 variables in the model statement were significant ($P = 0.0001$). When means were compared by week, high-dose birds had a significantly lower average hemoglobin concentration at 4 wk PI than either low-dose or control birds. Differences were not significant at other weeks PI. Differences in average hemoglobin concentration were often significant when means were compared by experimental group, however,

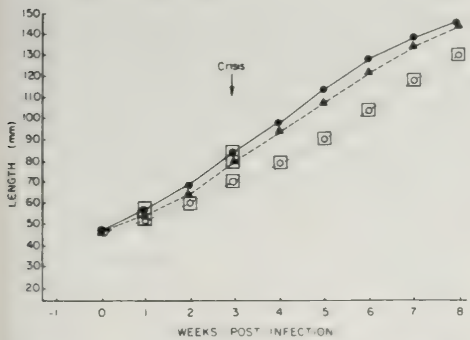


FIGURE 14. Average tarsometatarsal lengths for high-dose (O), low-dose (▲), and control (●) poults with experimental infections of *H. meleagridis*. Points surrounded by a square indicate significantly different means when comparisons are made among the 3 experimental groups by week, $P < 0.05$.

FIGURE 16. Average hemoglobin values for high-dose (O), low-dose (▲), and control (●) poults with experimental infections of *H. meleagridis*. Points surrounded by a square indicate significantly different means when comparisons are made among the 3 experimental groups by week, $P < 0.05$.

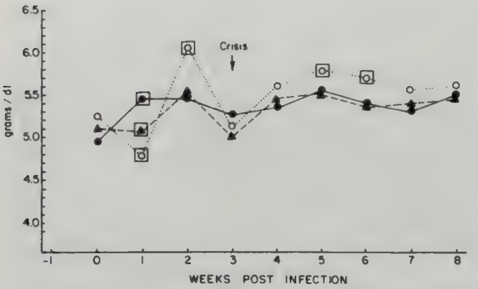


FIGURE 17. Average plasma protein concentrations for high-dose (○), low-dose (▲), and control (●) poults with experimental infections of *H. meleagridis*. Points surrounded by a square indicate significantly different means when comparisons are made among the 3 experimental groups by week, $P < 0.05$.

all 3 groups showed similar increases or decreases at each week with the exception of high-dose birds at 4 wk PI (Fig. 16).

Plasma protein concentration

Statistical analysis of plasma protein concentration revealed that all variables in the model statement were significant ($P < 0.0125$). When means were compared by week, all 3 groups were significantly different at 1 wk PI. Control birds had the highest average plasma protein concentration and high-dose birds had the lowest. By 2 wk PI, the average plasma protein concentration was significantly greater for high-dose birds than either low-dose or control birds. Means for all groups were not significantly different at 3 and 4 wk PI. At 5 and 6 wk PI, high-dose birds had an average plasma protein concentration that was significantly higher than that of low-dose and control birds. Means were not significantly different at 7 and 8 wk PI (Fig. 17). When average plasma protein concentrations were compared by experimental group, considerable overlap was detected. Low-dose birds had significantly lower average plasma protein concentrations at 0, 1, and 3 wk PI than they did at 2, 4, 5, 6, 7, and 8 wk PI. High-dose birds had significantly lower average plasma protein concentrations at 1 and 3 wk PI than at 0, 4, 5, 6, 7, and 8 wk PI. Control birds had a significantly lower average plasma protein concentration at week 0 PI than at any other week PI (Fig. 17).

DISCUSSION

Experimental infections of *Haemoproteus meleagridis* produced lameness and a moderate to severe myositis that was associated with the development of intramuscular megaloschizonts. These megaloschizonts were morphologically identical to second-generation megaloschizonts of *H. meleagridis* described by Atkinson et al. (1986) in experimentally infected domestic turkeys. In addition, small, thin-walled splenic schizonts that contained small, spherical pre-erythrocytic merozoites were found in 1 high-dose bird. Splenic schizonts that contained long, slender merozoites, morphologically similar to first-generation merozoites described by Atkinson et al. (1986), were also observed. The presence of these long, slender merozoites on day 19 PI and the occurrence of a smaller second peak in parasitemia in both low- and high-dose groups on day 38 PI indicates that at least some first-generation merozoites may be capable of initiating multiple generations of schizogony and serve as a source for gametocytes in chronic or relapsing infections. Other studies have reported the development of exoerythrocytic schizonts of *Plasmodium* and *Leucocytozoon* in reticular cells of the spleen (Huff, 1969; Akiba et al., 1971). Host cell nuclei of the splenic schizonts were not hypertrophied as occurs in *Leucocytozoon* infections.

Reductions in growth and weight gain in experimentally infected poults were dose dependent and most pronounced between 1 and 3 wk PI during development of megaloschizonts (Figs. 13, 14). The onset of lameness and anorexia in the high-dose birds was approximately 1 wk later than in experimental infections described by Atkinson et al. (1986) and was probably associated with the inflammatory response to developing megaloschizonts. Inflammatory changes associated with rupture of the first-generation schizonts between 5 and 8 days PI may not have been as severe as those described by Atkinson et al. (1986) because high-dose birds were infected with fewer than ⅓ as many sporozoites.

Following the crisis, all infected birds improved. This was most evident among turkeys in the high-dose group that exhibited little significant weight gain between 0 and 2 wk, between 1 and 3 wk, and between 3 and 4 wk PI (Fig. 13). The surviving high-dose birds remained significantly smaller than control and low-dose turkeys throughout the course of the study (Fig. 14). Low-dose birds were not significantly smaller than controls.

Few host effects were associated with the development of the erythrocytic gametocytes, al-

though these may have been masked by the massive host response to the megaloschizonts. Average hematocrit and hemoglobin values were not significantly different for any of the 3 experimental groups at the crisis, or at the second peak in parasitemia at 6 wk PI (Figs. 15, 16). A significant fall in average hemoglobin concentration occurred in the high-dose group, 1 wk after the crisis. This drop corresponded to the clearance and replacement of parasitized red blood cells with immature erythroblasts that had not completely synthesized their total hemoglobin content (Lucas and Jamroz, 1961). The absence of other significant weekly differences in average hemoglobin concentration and average hematocrit among the 3 experimental groups indicates that removal of parasitized red cells was balanced by the differentiation and release of erythroblasts.

Deposition of pigment in macrophages of the spleen, liver, and lungs began at approximately day 22 PI, when maturing gametocytes began to develop detectable pigment granules. The clearance of parasitized erythrocytes from the circulation was probably accomplished by phagocytic activity of these cells, as has been described in infections of *Plasmodium* (Taliaferro, 1941). Most pigment was deposited in the spleen, where the major elimination of the parasite population from the peripheral circulation occurs (Taliaferro, 1941). Follicular hyperplasia and enlargement of the spleen, characteristic of other species of *Haemoproteus* and *Plasmodium,* also occurred (Taliaferro, 1941; Russell et al., 1943; Becker et al., 1956).

The significant decrease in plasma protein levels in the low-dose and high-dose birds at 1 wk PI may have been related to the increase in vascular permeability that accompanies acute inflammatory responses (Fig. 17) (Smith et al., 1972). Atkinson et al. (1986) observed an acute inflammatory response to the rupture of first-generation schizonts between 5 and 8 days PI, which corresponds closely to the significant decrease in plasma protein levels in this study. The increase in plasma protein concentrations in the high-dose group at 2 wk PI may have been related to dehydration observed among these turkeys (Fig. 17). Augustine (1982) noted similar increases in plasma protein concentrations when turkeys were deprived of water for periods of up to 72 hr. The increase in average plasma protein concentration at 5 and 6 wk PI may reflect the synthesis of immunoglobulins (Fig. 17). Other studies of avian species of *Plasmodium* and *Leucocytozoon* have documented increases in parasite-specific immunoglobulins as the infections progressed (Congdon et al., 1969; Morii, 1972).

Salmonella is frequently isolated from commercial feeds that use animal products to boost protein levels (Williams, 1978). It is likely that the turkeys acquired *Salmonella* from the unmedicated game bird chow used in the experiment. The close proximity of the battery cage compartments to one another, the inevitable fecal contamination that occurred in the food and water, and successful isolation of *Salmonella* from representatives of each experimental group suggests that all the birds were infected. Because birds vary in their output of *Salmonella* organisms from day to day, the low number of *Salmonella* isolations from cloacal swabs of each group at 4 and at 8 wk PI is not surprising (Williams, 1978). It is significant that the only birds to develop clinical salmonellosis were those in the high-dose group. They exhibited clinical signs of infection between days 12 and 28 PI when stress from the *H. meleagridis* infection reached its peak.

It is impossible to determine from our data whether the high (33%) mortality in the high-dose group resulted from the *H. meleagridis* infection alone, or whether concurrent *Salmonella* and *Aspergillus* infections were significant factors. The splenic atrophy observed in the 4 fatal infections suggests that a failure or severe depression of the immune system in these birds may have contributed to their early deaths. Studies of interactions between *P. berghei* and *Salmonella typhimurium* (Kaye et al., 1965) and *P. yoelii* and *Bordetella pertussi* (Viens et al., 1974) have shown that *Plasmodium*-infected mice with concurrent bacterial infections died earlier than mice infected with either agent alone. Cox (1978) suggested that the synergism between *Plasmodium* and other infectious agents may result from the immunodepression that often accompanies plasmodial infections. Similar studies have not been conducted with species of *Haemoproteus,* although other infectious agents, including *Salmonella,* are commonly found in wild turkeys with *Haemoproteus* infections (Roslien and Haugen, 1970; Forrester et al., 1974; White et al., 1981).

Considering how prevalent *Haemoproteus* is in many bird populations (Bennett et al., 1982), the few reports of pathological changes in infected birds have done little to refute the view

that avian species of *Haemoproteus* have little effect on their hosts (Levine, 1973; Fallis and Desser, 1977; Kemp, 1978; Bennett et al., 1982). The most thorough study of naturally acquired *Haemoproteus* infections was done by O'Roke (1930) in California valley quail. He attributed morbidity and mortality in birds infected with *H. lophortyx* to anemia caused by rupture of parasitized erythrocytes as the infected cells passed through the capillary beds. Significantly, results of this study demonstrated that the most pathogenic effects of *H. meleagridis* infection were associated with preerythrocytic stages of development rather than with the circulating gametocytes.

Recently Miltgen et al. (1981) and Atkinson et al. (1986) have suggested that megaloschizonts of several organisms of uncertain taxonomic status, including *Arthrocystis galli,* may actually be species of *Haemoproteus*. Reports by Levine et al. (1970), Garnham (1977), Opitz et al. (1982), and Gardiner et al. (1984), have documented localized epizootics in California quail, chickens, and parakeets where infected birds exhibited a severe myositis in association with the developing megaloschizonts. The authors noted extensive muscle necrosis, inflammation, and hemorrhage around the megaloschizonts. Opitz et al. (1982) also observed dystrophic calcification of necrotic muscle fibers and formation of scar tissue. In one report, pigmented, *Haemoproteus*-like gametocytes were present in the peripheral circulation (Gardiner et al., 1984). Genetic differences between domestic and wild turkeys make it difficult for us to draw any conclusions about the potential impact of *Haemoproteus* infections on wild turkeys. It is significant, however, that Atkinson and Forrester (1987) have described a similar myopathy in association with intramuscular megaloschizonts of *H. meleagridis* in a naturally infected wild turkey. The possibility that *H. meleagridis* may be a cause of morbidity and mortality in wild turkeys in holoendemic areas such as southern Florida where rates of transmission are high (Atkinson et al., 1988) deserves further study.

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THE EFFECTS OF CROWDING ON ADULTS OF *ECHINOSTOMA REVOLUTUM* (DIGENEA: ECHINOSTOMATIDAE) IN EXPERIMENTALLY INFECTED GOLDEN HAMSTERS, *MESOCRICETUS AURATUS*

José Franco, Jane E. Huffman*, and Bernard Fried†
Department of Zoology and Physiology, Parasitology Laboratory, Rutgers—The State University, Newark, New Jersey 07102

ABSTRACT: All 30 female golden hamsters, *Mesocricetus auratus*, fed either 125 ± 50 (group A), 300 ± 50 (group B), or 500 ± 50 (group C) metacercarial cysts of *Echinostoma revolutum* were infected 7–35 days postexposure. The mean number of worms in A, B, and C were 62, 96, and 212, respectively. Most of the worms in A were in the jejunum, but in C worms were about equally distributed in the duodenum, jejunum, and ileum, and some were in the cecum. The body area and wet and dry weights of worms from C were significantly less than that of A or B at 2, 4, and 5 wk postinfection. *Echinostoma revolutum* eggs were in the feces of 100% of the hamsters by days 12, 13, and 14 in A, B, and C, respectively.

The growth and development of *Echinostoma revolutum* (Froelich, 1802) in natural and experimentally infected avian and mammalian hosts was studied by Beaver (1937). Senger (1954) studied the growth, development, and survival of *E. revolutum* in domestic chicks and rats. The infectivity, growth, and development of *E. revolutum* in the golden hamster has been reported (Franco et al., 1986).

There are relatively few experimental studies on the effects of intraspecific crowding of digeneans (Willey, 1941; Fried and Nelson, 1978; Mohandas and Nadakal, 1978; Nollen, 1983; Fried and Freeborne, 1984). In the present study we examine growth and maturation, fecundity, and niche of *E. revolutum* at various intensities in the golden hamster (*Mesocricetus auratus*).

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* Author to whom correspondence should be addressed at Department of Biological Sciences, East Stroudsburg University, East Stroudsburg, Pennsylvania 18301.

† Department of Biology, Lafayette College, Easton, Pennsylvania 18042.

MATERIALS AND METHODS

Metacercarial cysts of *E. revolutum* were removed from the renal tissue of laboratory infected *Physa heterostropha* snails (Franco et al., 1986). Cysts, 125 ± 25 (group A), 300 ± 50 (group B), or 500 ± 50 (group C), were administered in distilled water per os, to 30, 5-mo-old female golden hamsters, 10 hamsters per group.

Hamsters were given food and water *ad libitum* and necropsied on days 7, 14, 21, 28, and 35 postinfection (PI). Hamsters were weighed prior to infection and on days 7, 14, 21, 28, and 35 PI. Feces were examined from day 8 to 14 PI for the presence of worm eggs.

The small intestine was removed and measured from the pylorus to the cecum and divided into 3 equal segments. The cecum was also examined separately and all worms recovered were placed in 0.85% saline. Ten worms from each group were selected for wet weight measurements on days 7, 14, 21, 28, and 35 PI and an average wet weight determined. Worms were then dried at 56 C for 24 hr and dry weights were determined. Ten worms from each group were fixed in hot (80 C) alcohol–formalin–acetic acid (AFA) on days 7, 14, 21, 28, and 35. The worms were stained in Gower's carmine, dehydrated, cleared in xylene, and mounted in Permount. Body length and midacetabular width measurements were made with the aid of a calibrated ocular micrometer.

Duncan's multiple range test was used along with the MANOVA test which includes Wilk's criterion, Pillai's trace, Hotelling–Lawley trace, and Roy's max-

TABLE I. *Infectivity and distribution of* Echinostoma revolutum *in the golden hamster.*

Group*	No. of cysts/host	Range and mean of worms recovered	Total no. (%) of worms recovered	No. of worms (%) in segments I, II, and III of the small intestine and in the cecum			
				I	II	III	Cecum
A	125 ± 25	7–137 (61.8)	618 (49.4)	164 (26)	307 (50)	143 (23)	4 (1)
B	300 ± 50	1–247 (96.2)	962 (32.1)	257 (26)	385 (40)	316 (33)	4 (1)
C	500 ± 50	90–384 (211.5)	2,115 (42.3)	718 (34)	754 (36)	487 (23)	156 (7)

* Groups A, B, and C each with 10 hamsters.

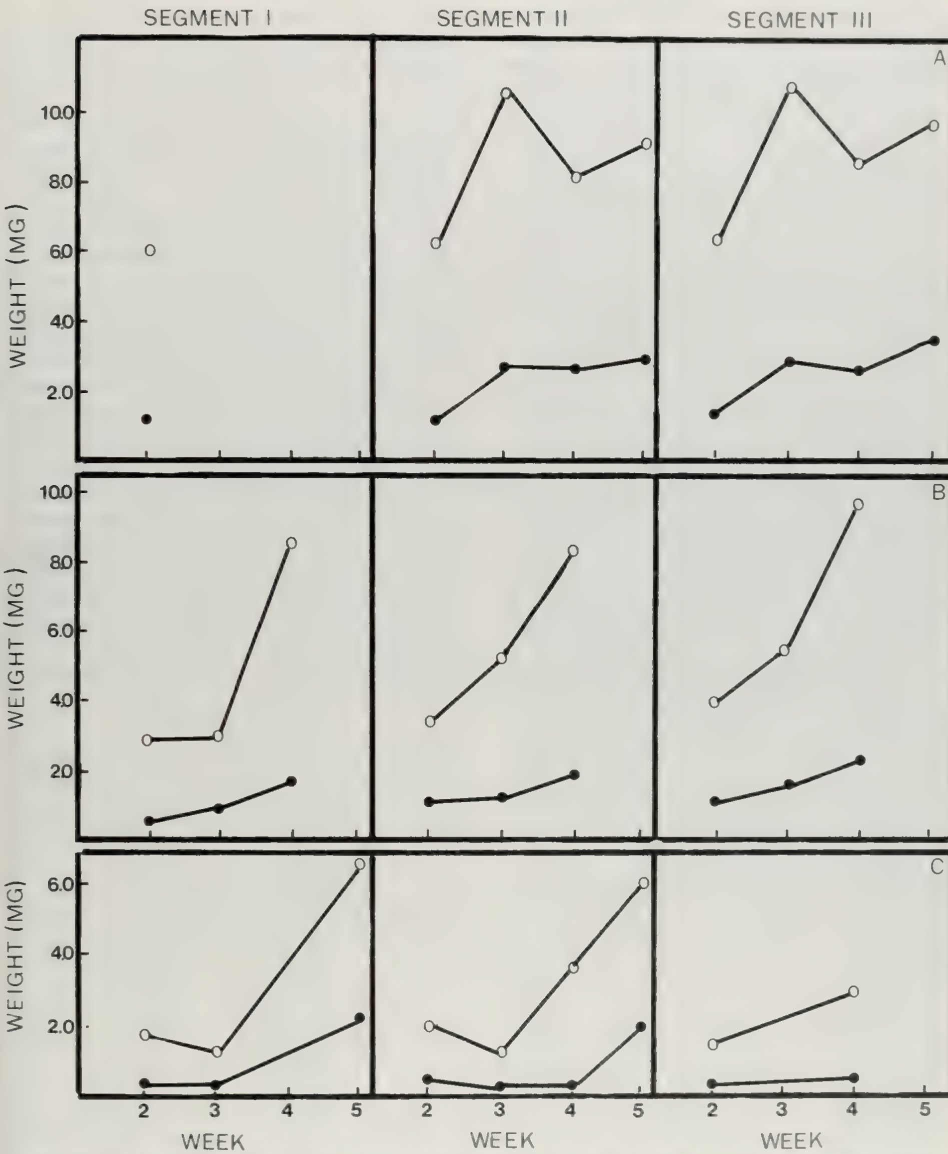

FIGURE 1. Mean wet (○) and dry (●) weights of *Echinostoma revolutum* adults by intestinal segment. Groups A, B, and C.

imum root criterion to indicate significant differences in worm body area between groups A and C. A value of $P < 0.05$ was considered significant.

RESULTS

Prevalence was 100%, intensity increased with increased dosage (Table I). Worms from group A were preovigerous up to day 8 postinfection. In these worms the diameter of the acetabulum was about 3 times that of the oral sucker. The acetabulum was located in the middle third by day 3, in the anterior half at day 6, and in the anterior third by day 9. In 5–9-day-old worms, the cirrus sac, uterus, ovary, ootype, and testes

TABLE II. *Percent golden hamsters with* Echinostoma revolutum *eggs in feces.*

	Day postinfection					
Group	9	10	11	12	13	14
A	0	50	88	100	100	100
B	0	50	75	88	100	100
C	0	25	38	67	80	100

were visible. Vitellaria were seen in some of these worms by day 8. Eggs were seen in some worms by day 9. Development occurred at a slower rate in worms from groups B and C (Table II).

Worms were found along the length of the small intestine and in the cecum. They occurred singly, in pairs, or in clusters, and were attached with the acetabulum to the host mucosal surface. Worms from groups B and C were found in all 3 intestinal segments throughout the course of the study, whereas worms from group A were only found in segment I during week 2 of the experiment (Fig. 1).

Mean wet and dry weights of worms from intestinal segments I, II, and III from 2 to 5 wk PI are shown in Figure 2. Wet weights were about 3 times greater than dry weights. Wet weights and dry weights were lower in the parasites from groups B and C than those from group A. Body area (length × midacetabular width) (Franco et al., 1986) is shown in Figure 2. These data were then used for statistical analyses. Significant differences occurred between the dose groups as time progressed as determined by Duncan's multiple range test. Significance occurred between the dose groups during weeks 1, 2, 4, and 5. No significance occurred during week 3. Three of the 4 MANOVA tests indicated where the significance occurred between the groups. Wilk's criterion, Pillai's trace, and Hotelling–Lawley trace indicated significance, whereas Roy's maximum root criterion indicated no significance. The MANOVA test indicated that in week 1, group B was significantly different in body area from groups A and C. In week 2, groups A and B were significantly different from group C. No significance occurred during week 3 among the dose groups. All 3 dose groups were significantly different in body area from each other during week

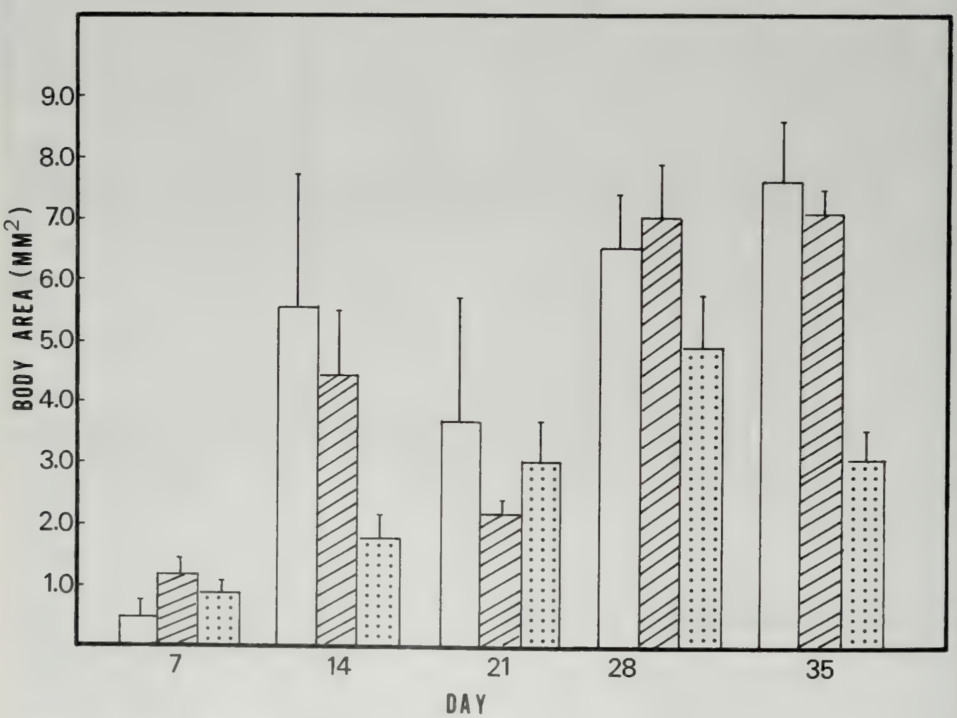

FIGURE 2. Mean body areas (mm²) ± SE of *Echinostoma revolutum* adults, 7–35 days old; group A (□), group B (▨), and group C (⬚).

4. Group C was significantly different from groups A and B during week 5.

DISCUSSION

The present study reveals that in *E. revolutum,* increased dosage levels and thus infrapopulation size influences several aspects of the parasite's development in the host intestine. It extends the maturation time and reduces total weight gain and overall body size, while enlarging the spatial distribution of the parasites in the gut. These all may be the product of a crowding effect facilitated by some form of intraspecific competition. Mohandras and Nadakal (1978) found that crowding reduced the length of *Echinostoma malayanum* in rats. Similarly, Fried and Nelson (1978) found that chickens infected with single adults of *Zygocotyle lunata* grown in the ceca were twice as long as worms from infections of 30 or more. It has been suggested that chemical "factors" released by worms associated with crowding may be important influences on worm growth (Fischthal et al., 1982; Roberts and Insler, 1982). The *E. revolutum*–hamster system used in the present study may be a convenient model to address this question experimentally.

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EVALUATION OF A POSSIBLE FUNCTIONAL RELATIONSHIP BETWEEN CHEMICAL STRUCTURE OF INTESTINAL BRUSH BORDER AND IMMUNITY TO *TRICHINELLA SPIRALIS* IN THE RAT

Yael Harari and Gilbert A. Castro

Department of Physiology and Cell Biology, University of Texas Medical School at Houston, Houston, Texas 77030

ABSTRACT: Primary exposure to *Trichinella spiralis* in the rat, while immunizing against reinfection, induces changes in the carbohydrate structure of intestinal brush border membranes. Immunity is expressed in heightened resistance to mucosal invasion by L_1 larvae, and the change in structure is evident in reduced membrane binding of the lectin, wheat germ agglutinin. The possibility that altered membrane composition is a requisite for expression of immunity was hypothesized and this was evaluated by correlating the maximum, specific binding of wheat germ agglutinin by isolated brush border membranes with (1) the expression of immunity acquired passively through serum transfer, and (2) the loss of immunity acquired from serial infections terminated in the intestinal phase. The hypothesis was further evaluated by determining whether the change in membrane structure represents a stimulus-specific response. We observed that (1) passively acquired immunity was not associated with a reduction in lectin binding and (2) short-term exposure to the intestinal stages of *T. spiralis* led to a reduction in lectin binding that was detectable at a time when rats were incapable of resisting reinfection. The change in lectin binding associated with trichinosis also accompanied infection with *Nippostrongylus brasiliensis*. Results uniformly support the conclusion that immunity to *T. spiralis* is independent of brush border membrane changes reflected in reduced binding of wheat germ agglutinin.

Intestinal brush border membranes (BBM) from rats infected with *Trichinella spiralis* show a reduced capacity to bind the lectin, wheat germ agglutinin (WGA). This reduction is maximally expressed as early as 2 wk after inoculation of hosts with L_1 larvae and remains so for at least 12 wk thereafter (Castro and Harari, 1982). The failure of BBM to bind WGA at the same level as membranes from uninfected, control rats is associated with a decrease in accessible sialic acid residues (Harari and Castro, 1983) that, along with N-acetylglucosamine, specifically bind WGA. In another study of BBM from infected rats, the transmethylation of phospholipids was quantitatively altered as compared with BBM from uninfected hosts (Harari and Castro, 1985). Inasmuch as an initial infection primes the host to resist reinfection with L_1 larvae, we hypothesize that observed changes in epithelial membranes may be a manifestation of alterations in epithelial cells that are induced by a primary infection and are present at the time of reexposure to the parasite. Furthermore, such alterations may interfere with larval infectivity upon subsequent challenge.

The objective of this paper was to test our hypothesis by determining whether (1) passively acquired immunity is associated with a reduction in BBM binding of WGA, (2) loss of infection-induced immunity associates with the expression of normal levels of WGA binding by BBM, and (3) the change in BBM binding of WGA induced by primary infection is unique to the *Trichinella*–rat model. Assuming that immunity and BBM changes expressed as a reduction in lectin binding are directly related phenomena, a positive correlation between the 2 events plus specificity of the response would be taken as support for our hypothesis.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, Pennsylvania) were used as experimental hosts. The strain of *T. spiralis* used is one that has been maintained continuously in our laboratory for several years by serial passage through CF-1 mice. Rats were infected orally with L_1 larvae obtained by the method of Castro and Fairbairn (1969a). Adult worms were collected from the intestine and counted as described previously (Castro and Fairbairn, 1969b). Some rats were infected with a strain of *Nippostrongylus brasiliensis* that was acquired from Dr. Lillian Mayberry, University of Texas at El Paso. Infective (L_3) *N. brasiliensis* larvae were collected from fecal cultures and stored at 25 C in Ringer–Locke solution according to Kassai (1982). Rats were injected by subcutaneous administration of 2×10^3, 10–20-day-old larvae. All rats were housed individually and given food and water *ad libitum*.

Sera

Immune serum for passive transfer was obtained as follows. Rats (75–100 g) were infected with 4.5×10^3

T. spiralis larvae, orally. Each rat was inoculated with 8×10^3 larvae 6–8 wk later. Six days later rats were anesthetized with an intramuscular injection of xylazine (Rompun®, Haven-Lockhart, Shawnee, Kansas), 5 mg/kg body wt., and ketamine hydrochloride (Ketaset®, Bristol Laboratories, Syracuse, New York), 75 mg/kg, and blood was collected by cardiac puncture. Control serum was collected from age-matched, nonimmunized (uninfected) rats.

Passive cutaneous anaphylaxis assay (PCA)

Immune sera were tested for anti-*Trichinella* antibodies using a passive cutaneous anaphylaxis assay (PCA). Different dilutions of test sera were injected intradermally in a volume of 0.1 ml on the backs of uninfected rats. Forty-eight hours later (Watanabe and Ovary, 1977), 2 mg of *Trichinella* antigenic protein were injected intracardially with 0.5 ml of 1.5% Evans blue dye in phosphate-buffered saline. Thirty minutes after antigen injection, the dimensions of the resulting blue zone on the deep surface of the skin were measured. The titer was defined as the highest dilution of serum that produced a blue spot greater than 5 mm in diameter.

Brush border membrane (BBM) isolation

BBM were obtained from rat small intestinal mucosa following the method of Kessler et al. (1978). The small intestine was removed from an anesthetized rat and rinsed in 0.9% ice-cold saline. The mucosa was scraped off with a glass slide. The scraped mucosa was suspended in 40 ml of 100 mM mannitol/1 mM Tris–HEPES/0.1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.5, and homogenized in a Waring blender at full speed for 3 min at 4 C. Solid $CaCl_2$ was added to the homogenate to a final concentration of 10 mM. The mixture was stirred for 20 min at 4 C. The suspension was then centrifuged at 3,000 *g* for 15 min. The supernatant fluid was collected and centrifuged at 27,000 *g* for 30 min. The resulting pellet was resuspended in 20 ml of 100 mM mannitol/2 mM Tris–HEPES/1 mM EDTA/0.1 mM PMSF, pH 7.5, and stirred at room temperature for 20 min. The suspension was centrifuged at 27,000 *g* for 30 min. The pellet was then resuspended in 3 ml of 100 mM mannitol/1 mM Tris–HEPES, pH 7.4, and stored in aliquots at −80 C until used. Protein content of BBM was measured according to Lowry et al. (1951) using bovine serum albumin as a standard.

Wheat germ agglutinin (WGA) binding to BBM

BBM (75 μg of protein) were incubated in glass tubes at 24 C for 60 min in a total volume of 0.2 ml phosphate-buffered saline containing 1% (w/v) fatty acid free bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Missouri), pH 6.0. WGA (Calbiochem, La Jolla, California) custom iodinated (ICN, Chemical and Radioisotope Division, Irving, California) with a specific activity of 20–30 Ci/g, was added in different amounts.

Following incubation, 5.0 ml of ice-cold phosphate saline buffer was added and the content of each tube was poured through GF/C Whatman filters (Whatman, Inc., Clifton, New Jersey) on a Millipore® (Millipore Corp., Bedford, Massachusetts) vacuum sampling manifold. Filters were washed twice with 5.0 ml of the cold buffer. The filters were then monitored for radioactivity in a Searle (Model 1195) gamma counter. Lectin binding was expressed in terms of specific binding, i.e., the amount of ^{125}I-WGA bound to BBM that could be displaced by adding N-acetylglucosamine and N-acetylneuraminic acid (2 mM) to the labeled lectin before the addition of the membrane protein. Calculations of molar concentration of WGA were based on a molecular weight of 18,000 (Lis and Sharon, 1977). The experiments carried out to establish appropriate conditions for measuring maximum binding (B_{max}) have been published (Castro and Harari, 1982).

Experimental design

Our initial experiment involved the transfer of resistance to infection to determine whether changes in epithelial BBM structure, as detected by membrane binding of WGA, is a requisite for expression of passively acquired immunity. Immune and nonimmune sera were administered intraperitoneally in a volume of 6 ml to 150–200-g anesthetized rats. Four days later rats were inoculated orally with 8×10^3 *T. spiralis* larvae to test for the expression of larval rejection. Worms were recovered from the small intestine 24 hr postinoculation (PI) to determine the effect of sera on worm establishment. From another group of recipients of serum, BBM were obtained 4 days after serum transfer according to the procedure described above. The capacity of these membranes to bind WGA was determined. Another experiment was carried out based on the prediction that if immunity to reinfection is directly related to events reflected in reduced binding of WGA by BBM, then membranes from rats in which immunity is known to decay should have a binding capacity for WGA indistinguishable from that measured in unimmunized (uninfected) rats. Infections were prevented from going full course by the administration of the anthelmintic, methyridine (2-beta-[methoxyethyl] pyridine) purchased from ICN Biochemicals, Inc., K&K Labs (Plainview, New York). The efficacy of methyridine in terminating infection in the intestinal phase has been definitively established by several investigators (Campbell et al., 1963; Denham, 1965; Despommier et al., 1974; Bell and McGregor, 1979) using various regimens. Important to the design in our experiment is knowledge that by allowing intestinal stages of the parasite to develop while curtailing the production and establishment of extraintestinal stages, an immunity develops (Campbell et al., 1963), which, in the rat, disappears within about 30 days after the immunizing inoculation (Bell and McGregor, 1979). This is in contrast to long-lasting immunity induced by an infection allowed to proceed full course (Bell and McGregor, 1979; Bullick et al., 1984). Rats (75–100 g) were inoculated orally with 1.5×10^3 *T. spiralis* larvae. On days 2 and 3 PI, methyridine (500 mg/kg body wt.) was administered intramuscularly to each rat. This protocol for infection and drug treatment was repeated twice. The infectivity of each batch of larvae used was established by recovering worms from a randomly selected rat. Control groups included rats that received the 3 immunizing doses of larvae but without drug follow-up, and rats that were placed on the drug regimen without being infected. BBM were collected from

TABLE I. *Maximum binding (B_{max}) of wheat germ agglutinin by intestinal brush border membrane from recipients of anti-*Trichinella *serum.*

B_{max}* (fmol bound mg^{-1} protein)		Difference (P value)
Immune serum†	Normal serum	
640 ± 130 (6)‡	460 ± 80 (5)‡	>0.20

* Brush border membranes isolated from rats 4 days after serum transfer.
† Anti-*Trichinella* PCA titer >256.
‡ Values are means ± SE (n). Difference between the 2 groups is not statistically significant as determined by Student's t-test for unpaired values (Snedecor and Cochran, 1980).

rats 70 days after the last treatment period and from control rats at the corresponding time. All batches of membranes were assayed for WGA binding.

Intestinal BBM were isolated from rats infected with *N. brasiliensis* to determine whether the change in BBM binding of WGA induced by *T. spiralis* was specific with regard to parasite stimulus. Membranes were obtained from rats killed 36–41 days PI.

Statistics

Student's t-test was used to statistically compare mean values between 2 groups. In comparing mean values among more than 2 groups, variance analysis and Duncan's new multiple range test were utilized (Snedecor and Cochran, 1980).

RESULTS

The passive transfer of immune serum was effective in conferring resistance to reinfection with *Trichinella* L_1 larvae. Recipients of serum having an anti-*Trichinella* PCA titer of >256 harbored significantly ($P < 0.05$) fewer worms (1,174 ± 75; mean ± SE, n = 9) than recipients of PCA-negative (normal) serum (2,260 ± 136; mean ± SE, n = 10) when challenged 4 days after serum transfer. Results revealed no significant difference in the membrane B_{max} for WGA between recipients of immune serum as compared with recipients of normal serum. In fact, the B_{max} was higher for those rats receiving immune serum (Table I).

TABLE II. *Maximum binding (B_{max}) of wheat germ agglutinin by brush border membrane from rats given abbreviated infection with* T. spiralis.

Treatment	No. animals	B_{max}* (fmol WGA bound·mg^{-1} protein)	P < 0.05†
Infection + methyridine	7	75 ± 20	
Infection	5	110 ± 12	
Methyridine	4	246 ± 29	
Uninfected	4	329 ± 70	

* Values are means ± SE.
† Differences between means compared by the Duncan multiple range method (Snedecor and Cochran, 1980). Any 2 means not spanned by the same vertical line are significantly different; any 2 spanned by the same line are not statistically different.

TABLE III. *Maximum binding (B_{max}) of wheat germ agglutinin by intestinal brush border membrane from rats infected with* N. brasiliensis.

B_{max}* (fmol WGA bound·mg^{-1} protein)		Difference (P value)
Infected	Uninfected	
97 ± 41 (7)	280 ± 29 (9)	<0.001

* Values are means ± SE (n). Difference between the 2 groups was compared using the t-test for unpaired values (Snedecor and Cochran, 1980).

BBM from rats given chemically abbreviated infections had a reduced capacity to bind WGA (Table II) as compared with uninfected rats, despite similar levels of intestinal parasitism in both groups following a challenge infection. Rats primed by methyridine-abbreviated infection harbored 3,939 ± 815 (mean ± SE; n = 3) worms, whereas rats primed with methyridine alone had 4,267 ± 623 (n = 6) worms. Rats were challenged 60 days after the priming regimens and intestinal worms were collected 24 hr after challenge.

The reduced level of binding in rats given abbreviated infections was comparable to that of binding to BBM from rats undergoing complete infection (Table II). This reduction is concluded to be due to the brief, intermittent encounter with the parasite and not to methyridine treatment per se, because the B_{max} for rats receiving only methyridine had significantly higher levels of binding, being of the same magnitude as that measured for uninfected rats (Table II). The effectiveness of methyridine in clearing worms from the intestine was evident from the finding that rats examined 2 days after the last dose of drug were free of intestinal parasites, whereas undrugged rats all harbored intestinal-stage parasites. Furthermore, rats given chemically abbreviated infections harbored no larvae in tongue, triceps, and diaphragm muscles when killed 60 days after the priming regimen.

Brush border membranes isolated from rats infected with *N. brasiliensis* bound significantly less WGA than those from uninfected controls (Table III). B_{max} was determined on BBM isolated 36–40 days after inoculation with infective larvae.

DISCUSSION

Resistance to reinfection with *T. spiralis* in the rat is a well-recognized phenomenon. However, the specific cellular and subcellular events in-

volved in preventing the establishment of L_1 larvae are not known. Our study addresses this issue. As a working hypothesis we proposed that the chronically expressed reduction in intestinal BBM sialic acid content following primary infection represents a physiological correlate of resistance to reinfection.

The possibility that worm infectivity might be impaired by chemical changes on host cell surface membranes, such as we observed, is supported by evidence derived from other host–parasite systems. Intracellular protozoans, for example, are known to elicit selective changes in target cell membrane structure and also to require carbohydrate receptors to establish parasitism. The surface membrane of erythrocytes infected with *Plasmodium knowlesii* becomes deficient in both D-galactose and α-mannosyl hydroxyl groups (Trigg et al., 1977; Shakespear et al., 1979; Vincent and Wilson, 1980). However, it is not known whether these changes have a significant influence on the host–parasite relationship. On the other hand lectin–carbohydrate interactions between the surface membranes of fibroblasts and *Trypanosoma cruzi* appear to mediate intracellular entry of the parasite (Henriquez et al., 1981). In similar fashion mannose/fucose receptors on monocyte-derived macrophages react with ligands on the surface of *Leishmania donovani* to promote ingestion of promastigote stages, a mandatory step for successful parasitism (Wilson and Pearson, 1986). The adherence of *Escherichia coli* to the mucosal surface of the intestine depends on the organism interacting with mannose receptors on epithelial cells (Ofek et al., 1977). Likewise, the attachment of *Giardia lamblia* to enterocytes purportedly is mediated by a ligand–carbohydrate interaction. A lectin on the surface of *G. lamblia* apparently becomes expressed when the parasite is exposed to a proteolytic enzyme such as trypsin. Appearance of this lectin allows the parasite to bind to mannose-6-phosphate receptors on the enterocyte surface (Lev et al., 1986). To our knowledge similar interactions of metazoan parasites with host cells at the membrane level, which might influence infectivity, are not known.

If enteric parasites depend on specific receptors, such as sialic acid, on epithelial cells to initiate infection, then reductions in these receptors might impede this process. However, such an assumption cannot be reconciled by results from our studies with *T. spiralis*. Evidence derived from 3 experimental designs supports the conclusion that immunity to reinfection is independent of changes in BBM carbohydrate structure reflected in reduced binding of WGA. The reductions in membrane B_{max} for WGA associated with immunity actively acquired through primary infection (Castro and Harari, 1982) was not expressed in passively immunized rats. Furthermore, BBM from rats exposed only to the intestinal stages of the parasite expressed reduced lectin binding without expressing resistance to reinfection. In this latter experiment both the lectin-binding assays and challenge infection were carried out 10 wk after exposing the hosts to methyridine-abbreviated infections. Failure to observe worm rejection at this time was expected, because rats immunized by infections terminated in the intestinal phase by treatment with methyridine express immune rejection of L_1 larvae for only 2–6 wk after the priming infections (Bell and McGregor, 1979). Despite the failure in our study of repeated, short-term exposure to *Trichinella* to confer an enduring immunity, such exposure was sufficient to induce a sustained reduction in BBM binding of WGA. Our measurements indicate the lack of a positive correlation between immunity and the presence of epithelial membrane changes in murine trichinosis. Additionally, the observation that infection with *N. brasiliensis* also reduced BBM binding of WGA indicates that this change may be a general response to enteric infection and not specific to trichinosis. Based on the strength of the collective evidence, we are compelled to reject our proposed hypothesis. In light of our conclusions other possibilities must be considered to explain the inability of *T. spiralis* L_1 larvae to effectively parasitize immune rats.

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LACK OF EFFECT OF UNMATED SCHISTOSOMES ON THE FECUNDITY OF MATED WORM PAIRS

Allen W. Cheever
Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT: An imbalance between the numbers of male and female worms had no significant effect upon the fecundity of *Schistosoma mansoni* in rhesus monkeys or upon the number of *S. mansoni* eggs in the tissues of infected persons or mice. The number of *S. japonicum* eggs in the tissues of infected rabbits was similarly unaffected by disproportion between worm genders.

An imbalance between the numbers of male and female *Schistosoma mansoni* has been shown to reduce the number of eggs per worm pair in the tissues of mice challenged with male or female worms (Harrison et al., 1982) and to be associated with decreased fecundity of worms in baboons examined 4–6 mo after a single infection (Damian and Chapman, 1983). I was concerned as to the effect of imbalance of worm genders on data from this laboratory and therefore reviewed data from several experiments using *S. mansoni* in mice or monkeys and *S. japonicum* in rabbits. Available data from humans were also reviewed.

MATERIALS AND METHODS

Rhesus monkeys (*Macaca mulatta*) were exposed percutaneously to 100 or 600 *S. mansoni* cercariae of a Puerto Rican strain (PR-1, Fletcher et al., 1981) and were killed 3 or 6 mo later (Cheever and Powers, 1969, 1972). Rabbits were examined 7 mo after exposure to 600 *S. japonicum* cercariae of a Philippine strain or 1 yr after exposure to 600 cercariae of a Japanese strain (Cheever et al., 1980a, 1980b). C57BL/KsJ mice were exposed to varied numbers of male and female cercariae of the PRC-3 strain of Puerto Rican *S. mansoni* (Fletcher et al., 1981). In one experiment, some of these mice were challenged with a large number of cercariae of the NAMRI strain (Stirewalt and Uy, 1969) of *S. mansoni*. All animals were killed by i.v. (monkeys and rabbits) or i.p. (mice) injection of pentobarbital.

Schistosomes were recovered from the portal venous system by perfusion as described elsewhere (Duvall and DeWitt, 1967; Cheever and Powers, 1969). The entire gut and representative portions of the liver were hydrolyzed in 4% KOH for 12–18 hr at 37 C and duplicate 1-ml samples were counted (Cheever, 1970). The number of eggs per worm pair in the tissues was calculated from the eggs in the liver and intestines and the number of mature worm pairs recovered. Generally, insignificant numbers of eggs were present in other tissues. Eggs in the feces of the monkeys were counted using Bell's technique (Bell, 1963), and eggs in the feces of rabbits by dilution of formalin-fixed feces or, if few eggs were present, by the quantitative formalin–ether technique of Knight et al. (1976). The fecundity of worms (N1, expressed as eggs per worm pair per day) in rhesus monkeys was calculated using the formula (from Cheever and Duvall, 1974)

$$N1 = \frac{0.693N}{T^{1/2}} + F$$

where N indicates the number of eggs per worm pair in the tissues, $T^{1/2}$ the half life in days of eggs in the tissues, and F the number of eggs per worm pair passed in the feces each day, generally the mean calculated from eggs found in 8 24-hr fecal collections made in the month before death. The $T^{1/2}$ is about 8 days in rhesus monkeys (Cheever and Powers, 1971). In mice, the number of tissue eggs per worm pair was used as a measure of fecundity and the feces were not examined. In rabbits and in humans, the effects of imbalanced gender were examined separately for tissue and fecal eggs; fecundity, as defined above, was not calculated, since no information is available concerning the $T^{1/2}$ of eggs in the tissues.

No monkeys or rabbits had a significant excess of female over male worms, i.e., none with a male : female ratio of <0.95. Thus, in these species, the effect examined in animals with an imbalance in worm genders was the effect of excess male worms. In mice, infections were given with an excess of male or female worms and the effects of each were examined.

The groups of animals above were chosen for analysis because a relatively large number of individuals was examined at a given time after infection and a wide range of ratios of male to female worms existed. Unpaired female worms were uniformly immature and tiny in all of these infections. None of the past experience of this laboratory has been deliberately omitted.

The techniques for worm recovery and for counting of eggs from infected persons have been described (Cheever, 1968; Cheever et al., 1977) and are not entirely comparable to those used for experimental studies. I think no systematic bias results when comparing human cases with each other.

I have examined the effect of worm gender on fecundity by regression of tissue eggs per worm pair, fecal eggs per worm pair, or fecundity (N1 as calculated above) on the ratio of worms of the predominant gender to worm pairs, i.e., by the male : female ratio or the female : male ratio, whichever was greater. This variable is referred to as the "gender ratio." Fecundity

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TABLE I. *Tissue eggs in C57BL/KsJ mice infected with excess male or female* S. mansoni *for 12 wk.*

Experiment and group	n*	Cercariae for infection	Worm pairs† ± SEM	Extra males* ± SEM	Extra females ± SEM	Eggs per worm pair ± SEM
1A‡	5	35 mixed genders	2.2 ± 0.7	0.7	0.2	7.8 ± 1.6
1B	4	35 mixed + 100 male	2.3 ± 0.6	17 ± 2	0	11.5 ± 1.4
1C	7	35 mixed + 300 male	3.0 ± 0.8	52 ± 9	0.3	6.5 ± 1.3
1D	7	35 mixed + 100 female	3.3 ± 0.6	0.4	22 ± 2	7.3 ± 1.3
1E	6	35 mixed + 300 female	3.0 ± 0.6	0.5	44 ± 9	8.1 ± 1.3
2A	11	44 mixed genders	3.6 ± 0.4	0.3	2.5	7.4 ± 0.6
2B	9	44 mixed + 100 male	6.8 ± 1.0	5 ± 1	0	6.6 ± 1.1
2C	11	44 mixed + 600 mixed at 8 weeks	4.5 ± 0.9	203§		7.7 ± 0.6

* n indicates the number of mice.

† Mean ± standard error of the mean.

‡ The number of eggs per worm pair in groups with balanced infections (1A and 2A) does not differ significantly from eggs per worm pair in the corresponding groups with excess male or female worms.

§ Total worms from second infection. The gender could not be determined in many of these.

in monkeys and rabbits is also presented for groups of animals with "gender balanced" or "gender imbalanced" infections, defining "gender imbalanced" infections as those with a male : female ratio of >1.20 after Damian and Chapman (1983).

RESULTS

The protocol and results for *S. mansoni*-infected mice are noted in Table I. Regression of tissue eggs per worm pair on worm pairs for the 60 mice in this experiment showed no significant relation between these variables. Regression of the extra number of male worms against eggs per worm pair in the intestines or total tissue eggs per worm pair showed no significant correlation with or without logarithmic transformation of the data. Similarly, the means of tissue eggs per worm pair showed no trends among the groups (Table I).

Data were available from 29 rhesus monkeys killed 3 mo after infection, including 14 infected with Puerto Rican strains of *S. mansoni* and the remainder with strains from St. Lucia, Brazil, and Tanzania (Cheever and Powers, 1969, 1971, 1972; Powers and Cheever, 1972). Fifteen monkeys were studied 6 mo after infection. Regression of tissue eggs per worm pair or calculated overall fecundity (eggs per day per worm pair) on worm pairs showed no relation of infection intensity (worm pairs) to eggs per worm pair at either time period. The ratio of male to female worms at 3 mo was also unrelated to worm fecundity or to tissue eggs by regression analysis or after calculation of mean values for animals with male : female ratios of <1.21, as opposed to those with more imbalanced infections (Table II). Means about other limiting ratios were tried unsuccessfully as well. At 6 mo after infection, a significant negative trend in fecundity was noted in monkeys with a larger ratio of males to females ($r = -0.55$, n = 14, $P < 0.05$, $t = 2.279$) on linear regression, but this trend depended almost entirely on a single "outlying" monkey (without which $r = -0.18$, $t = 0.62$, for n = 13). Division of the monkeys into groups in which worm genders were balanced or unbalanced did not show significant differences in worm fecundity (Table II).

TABLE II. *Worm fecundity in* S. mansoni-*infected rhesus monkeys examined at 3 or 6 mo.*

Group based on male : female ratio	n*	Worm pairs†	Tissue eggs per worm pair in 1,000's†	Fecal eggs per worm pair per day†	Fecundity per worm pair per day†
3-mo infections					
m:f < 1.21‡	11	112 ± 29	6.6 ± 0.7	189 ± 43	769 ± 96
m:f ≥1.21	18	124 ± 13	5.7 ± 0.4	196 ± 16	683 ± 42
6-mo infections					
m:f < 1.21	3	66 ± 28	6.0 ± 2.0	149 ± 61	667 ± 165
m:f ≥1.21	11	18 ± 4	4.5 ± 0.6	227 ± 36	614 ± 76

* n indicates the number of animals.

† Mean ± standard error of the mean.

‡ The only significant difference between "gender balanced" and "imbalanced" groups is that between numbers of worm pairs 6 mo after infection.

TABLE III. *Relation of tissue and fecal eggs to worm gender ratio in rabbits infected with* S. japonicum.

Duration of infection	n*	Male : female ratio	Worm pairs†	Tissue eggs per worm pair 1,000's†	Fecal eggs per worm pair per day†
7 mo	3	<1.21‡	34 ± 6	137 ± 30	53 ± 2
7 mo	8	>1.21	23 ± 9	106 ± 7	107 ± 52
1 yr	9	<1.21‡	59 ± 11	198 ± 47	157 ± 28
1 yr	11	≥1.21	44 ± 7	204 ± 39	266 ± 83

* n indicates the number of animals.

† Mean ± standard error of the mean.

‡ The mean tissue or fecal eggs per worm pair also did not differ if groups with a male : female ratio of <1.5 or >2.0 were compared to groups with a higher male : female ratio.

Similar analysis of *S. japonicum*-infected rabbits uncovered no significant correlations of male to female ratio with the number of eggs per worm pair in the tissues or feces by regression, and no substantial or significant differences were noted in the mean values for these variables (Table III). The ratio of male to female worms showed no significant trend when examined in relation to the number of worm pairs.

Data from *S. mansoni*-infected humans were available from Brazil (Cheever, 1968) and Egypt (Cheever et al., 1977). Regression of tissue eggs per worm pair from Brazilian cases on the gender ratio gave no significant trend ($n = 31$), nor were regressions of eggs per gram feces per worm pair on gender ratio significant ($n = 70$). Cases from which less than 5 worm pairs had been recovered generally had both high tissue eggs per worm pair and high ratios of male : female or female : male worms. Elimination of these cases from the analysis did not significantly affect the results. For Egyptian cases, the regressions also lacked significant slope for regression of tissue eggs on the "gender ratio" ($n = 58$) or eggs in the feces ($n = 19$). In both the Brazilian and the Egyptian material, the slopes of the regressions were of variable sign in the different groups considered, i.e., nothing vaguely resembling any trend was present (data not shown).

DISCUSSION

The analyses presented did not detect a significant effect of imbalance of worm gender on egg production by the worm pairs present in the same hosts. Previous attempts to demonstrate such an effect in *S. haematobium*-infected capuchin monkeys also failed (Cheever et al., in press). This contrasts with the demonstration of such an effect by Damian and Chapman (1983) in *S. mansoni*-infected baboons and by Harrison et al. (1982) in CBA mice. The present experiments with *S. mansoni* differed from those studies in experimental design, as well as in the strain of schistosome employed and the species or strain of host used.

The results indicate that imbalance of worm gender does not always affect egg production by the paired worms.

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SCHISTOSOMA MANSONI: CORRELATIONS BETWEEN MOUSE STRAIN, SKIN EICOSANOID PRODUCTION, AND CERCARIAL SKIN PENETRATION

A. C. Fusco, B. Salafsky, B. Ellenberger, and L.-H. Li

Department of Biomedical Science, University of Illinois College of Medicine at Rockford, 1601 Parkview Avenue, Rockford, Illinois 61107

ABSTRACT: We have previously reported that cercarial penetration is highly correlated with cercarial production of leukotrienes (LT's) and hydroxyeicosatetraenoic acids (HETE's). Because skin also produces various eicosanoids, we undertook an investigation of skin eicosanoids in various strains of mice and 1 strain of rat in order to ascertain if skin eicosanoids could be correlated to cercarial penetration. SENCAR, ICR, NMRI, A/J, C3H/HeJ, C57Bl/6, ASEBIC, and BALB/c mouse strains were used in this study as well as the SD-Rat strain. The ability of cercariae to penetrate skin was strain specific. A/J and SENCAR mice had the highest penetration rates (~98%), whereas the SD-Rat strain had the lowest (43%). These penetration rates we linearly correlated with tail skin HETE production at 10 min ($R = 0.826$), whereas HETE production at 60 min had a parabola-shaped relationship ($R = 0.793$). The primary infection of mice with *Schistosoma mansoni* cercariae may therefore be directly correlated with both the skin's innate ability to synthesize HETE, as well as with cercarial eicosanoid production, especially HETE levels. However, we believe that skin eicosanoid production is just one of many factors affecting cercarial skin penetration. Other factors discussed are: skin surface fatty acid levels, cercarial eicosanoid production, epidermal vs. dermal eicosanoid production, and the immunocompetence of the host.

We have recently reported that cercarial penetration is highly correlated with cercarial production of leukotrienes (LT's) and hydroxyeicosatetraenoic acids (HETE's) (Fusco et al., 1986), and LTB_4 (or one of its metabolites) appears to be the major leukotriene species associated with penetration (Fusco et al., 1987). We have also shown that approximately 60% of the eicosanoids produced by cercariae are being secreted (Salafsky and Fusco, 1987). Because the skin barrier is not inert, but is a biological entity capable of mounting a strong immunological defense against invasion (Bos and Kapsenberg, 1986), some type of cercaria : skin interaction might be expected. Examination of cercaria : host skin reactions using histological, electron microscopic, or autoradiographic techniques have shown remarkably little response to cercarial invasion at the morphological level (Wheater and Wilson, 1979; Mangold and Dean, 1983; Incani and McLaren, 1984). Given the immunomodulatory activity of various eicosanoid species, these facts strongly suggest that cercarial eicosanoid secretion may serve to mask local immunological reactions to this foreign body. Because such immunological events can be triggered or dampened by various eicosanoid species and both skin (epidermal and dermal tissues) and cercariae synthesize eicosanoids, the interactions between skin and cercarial eicosanoid production may be important in regulating the penetration process. However, such interactions are extremely difficult to experimentally dissect, because they are localized and relatively small compared to the mass of tissue involved. Thus, we decided to approach this question by examining the innate ability of tail skin to synthesize eicosanoids in relation to the ability of cercariae to penetrate tail skin (innate meaning the skin's ability to synthesize eicosanoids when an appropriate substrate is available).

Surprisingly, there is little information in the literature on the role of mouse strain in the *initial* penetration response. The majority of studies examining various mouse strains were concerned with adult worm recoveries and/or secondary infections in relation to host resistance (see Discussion). The study reported here demonstrates significant differences in cercarial penetration rates between mouse strains and relates these differences to skin eicosanoid production.

MATERIALS AND METHODS

Mouse strains

ICR mice and SD-Rats were purchased from King/Sasco (Madison, Wisconsin), SENCAR mice were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, Indiana), and A/J, C3H/HeJ, C57Bl/6, ASEBIC, and BALB/c strains were purchased from Jackson Laboratories (Bar Harbor, Maine). The NMRI strain was kindly donated by Lt. Col. R. D. Gunnels, Naval Medical Command, National Capital Region (Bethesda, Maryland). All mouse strains were 4–6 wk of age when

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used. The SD-Rat strain was infected between 21–24 days. Only male mice and rats were used in these studies. Mice were kept in groups of 4 or 5 in AAALAC (American Association of Accreditation of Laboratory Animal Care) approved quarters under controlled lighting conditions (12-hr cycle). They received food and water *ad libitum* and were kept on hardwood bedding (BetaChip, Northern Products Corporation, Warrenburg, New York). Bedding was changed daily.

Parasite maintenance

The life cycle of *Schistosoma mansoni* was maintained in our laboratory using *Biomphalaria glabrata* as the intermediate host and male ICR strain mice as the definitive host. Snails were maintained in total darkness and were exposed to light only during cercarial shedding. The *S. mansoni* strain was originally obtained from Dr. Yung-san Liang, University of Lowell Center for Tropical Diseases (Lowell, Massachusetts).

Radiolabeling of cercariae

Cercariae were radiolabeled using the method of Georgi (1982). Briefly, infected snails were exposed individually to 10 μCi ^{75}selenomethionine in 4 ml of spring water overnight. Three days later, all snails shed labeled cercariae (100%). Snails so treated can be shed approximately 3 times before cercarial labeling decreases below an acceptable level for autoradiographic analysis.

Infection of mice

All mice were exposed to an average of 75 radiolabeled cercariae at room temperature (21–23 C) via the tail route for 1 hr. Unanesthetized mice were placed in restraining chambers and their tails were placed into test tubes (16 × 100 mm) containing 10 ml of bottled spring water (Purity Bottling Co., Waukesha, Wisconsin) into which cercariae had been placed 5–15 min earlier. Within each exposure group, the number of cercariae was known within a mean and standard deviation of less than 3. Except for the ASEBIC strain in which 7 mice were used due to the difficulty of obtaining large numbers of this mouse strain, 11–13 mice were used to determine cercarial penetration. One or 2 groups of mice were infected at the same time; thus, different strains were exposed to different sheds and/or batches of cercariae. In order to minimize effects due to different cercarial sheds or batches, cercariae were shed at approximately the same time each day (10 A.M.), used within 2 hr of shedding, and snails were repeatedly shed only after a 3 day "rest." In addition, 3 strains (ASEBIC, NMRI, and SENCAR) were exposed on different days with different batches of cercariae, while the cercarial penetration rates for the ICR strain (the control strain used to maintain the life cycle in our lab) was routinely monitored and found to average 77% using the above infection protocol.

Autoradiography

Tail tissue was processed by stripping tail skin from mice, spreading it onto 8″ × 10″ cardboard blotters, covering each tail with cellophane tape, and finally covering the cardboard with plastic wrap. Approximately 10 tails were placed on each cardboard sheet. The cardboard sheets were sandwiched between paper towels and dried at 40 C for 24 hr. The tail side of the cardboard sheet was placed into direct contact with Kodak XAR x-ray film and placed into lead-backed Kodak x-ray exposure holders (#149-2776). Intensifying screens were not used. Exposure was for 2 wk at room temperature in a press to assure firm contact between tissue and x-ray film. Tails were processed at 24 hr post-exposure to cercariae.

Eicosanoid measurement in tail skin homogenates

Tail skin (*not* exposed to cercariae) was homogenized in 0.1 M phosphate buffer (pH 7.2), centrifuged to remove debris, and the supernatant collected. The protein homogenate was diluted to 1 mg protein/ml and brought to 3 mM linoleate (3 μCi ^{14}C-U-linoleate/7 ml) as a substrate for eicosanoid synthesis. After 10, 30, and 60 min of incubation at 37 C, 2-ml aliquots were removed, extracted for eicosanoids, and subjected to HPLC analysis of eicosanoid production. HPLC was via the method of Henke et al. (1984). Eicosanoid peaks were monitored directly from HPLC eluant using a Ramona D in-line liquid scintillation counter (In/Us Service Corporation, Fairfield, New Jersey) using BioCount (RPI, Mt. Prospect, Illinois) as the scintillant. Data were collected via analog output from the Ramona to a MetraByte Chrom I A/D data acquisition card (MetraByte Corporation, Taunton, Massachusetts) connected to an IBM PC using Labtech Notebook Data Acquisition Software (Laboratory Technologies Corporation, Wilmington, Massachusetts). Chromatographs were integrated and analyzed using Labtech Chrom. Each sample was replicated 5 times (ASEBIC—3 times) and averaged together using Lotus 123 (Lotus Development Corporation, Cambridge, Massachusetts) software. Total eicosanoid production and PG, LT, and HETE synthesis were measured by counting DPS recovered between retention times of 7–70 min, 7–24 min, 24–50 min, and 50–70 min, respectively. Identification of various HPLC peaks was done using external tritiated eicosanoid standards (PGE_1, PGE_2, LTB_4, LTC_4, 5-HETE, and 15-HETE).

Statistics

Curve fitting was performed using SigmaPlot software (Jandel Scientific, Sausalito, California), and ANOVA and 1-tailed *t*-test comparisons were performed using SPSS/PC (SPSS, Inc., Chicago, Illinois).

RESULTS

Cercarial penetration/migration vs. mouse strain

Because our laboratory maintains adult *S. mansoni* in ICR mice, this *Schistosoma* strain has become adapted to the ICR mouse; therefore, ICR mice were used as a control to which all other strains were compared. A definite relationship existed between cercarial penetration of mouse (or rat) tail skins and mouse strain (Fig. 1). Four penetration classes were statistically evident when compared to the ICR strain: SENCAR and A/J strains, which approached 100% penetration rates; ICR, C3H, NMRI, ASEBIC, and C57, which ranged from 65 to 80% pene-

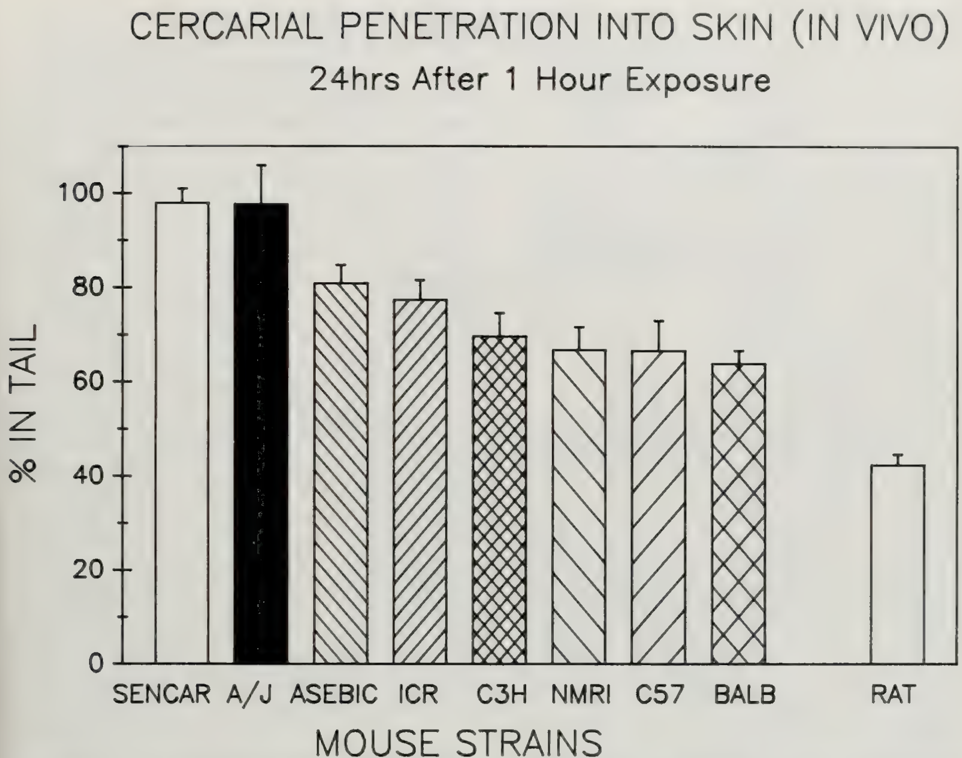

FIGURE 1. Cercarial penetration into skin (*in vivo*) 24 hr after an initial 1-hr exposure. Various mouse strains and 1 rat strain were exposed for 1 hr to an average of 75 cercariae labeled with ^{75}Se. Twenty-four hours later mice were sacrificed and their tail skin removed and subjected to autoradiography on Kodak XAR film for 2 wk. The data recorded represent the number of exposed foci on the x-ray film (i.e., penetrated cercariae) expressed as a percentage of the total number of cercariae used to infect the various strains. Error bars represent standard errors of 11–13 replications, except for ASEBIC mice in which only 7 replications were done.

tration rates; the BALB strain, which had a 63% penetration rate; and SD-Rat, which was 43%. Although the penetration rate in BALB strain was statistically different from ICR ($P = 0.01$), it was not statistically different from C3H, NMRI, and C57 strains. When the ICR strain was not used as a control and a 1-way ANOVA was performed on the data, there was a highly significant difference between strains (F probability < 0.0001). Duncan's Multiple Range Test identified 3 statistically significant groups at the $P = 0.05$ level of significance: the SD-Rat strain; the ICR, C3H, NMRI, ASEBIC, BALB, and C57 group; and the SENCAR and A/J group.

Mouse tail skin eicosanoid production vs. cercarial penetration

Total prostaglandins (PG's), leukotrienes (LT's), and hydroxyeicosatetraenoic acids (HETE's) in skin were measured at 10, 30, and 60 min for each mouse strain. Total PG's and LT's showed no correlation with cercarial penetration at 10, 30, and 60 min. However, overall LT production did show a linear relationship if 3 of the 9 strains (A/J, SENCAR, and SD-RAT) were omitted from the regression line. This line had an equation of $Y = -1.82 \cdot 10^{-3}X + 83.08$ ($r = 0.947$) at 10 min, $Y = -1.29 \cdot 10^{-3}X + 81.56$ ($r = 0.948$) at 30 min, and $Y = -9.10 \cdot 10^{-4}X + 79.84$ ($r = 0.84$). HETE levels correlated with cercarial penetration at 10 min and to a lesser extent at 60 min (Fig. 2). At 10 min a direct negative linear relationship was recorded between penetration and HETE levels ($Y = -3.52 \cdot 10^{-3}X + 102.14$, $r = 0.826$). Lower skin HETE levels were more favorable to penetration than higher levels. Hydroxyeicosatetraenoic acid production at 60 min showed a less favorable cor-

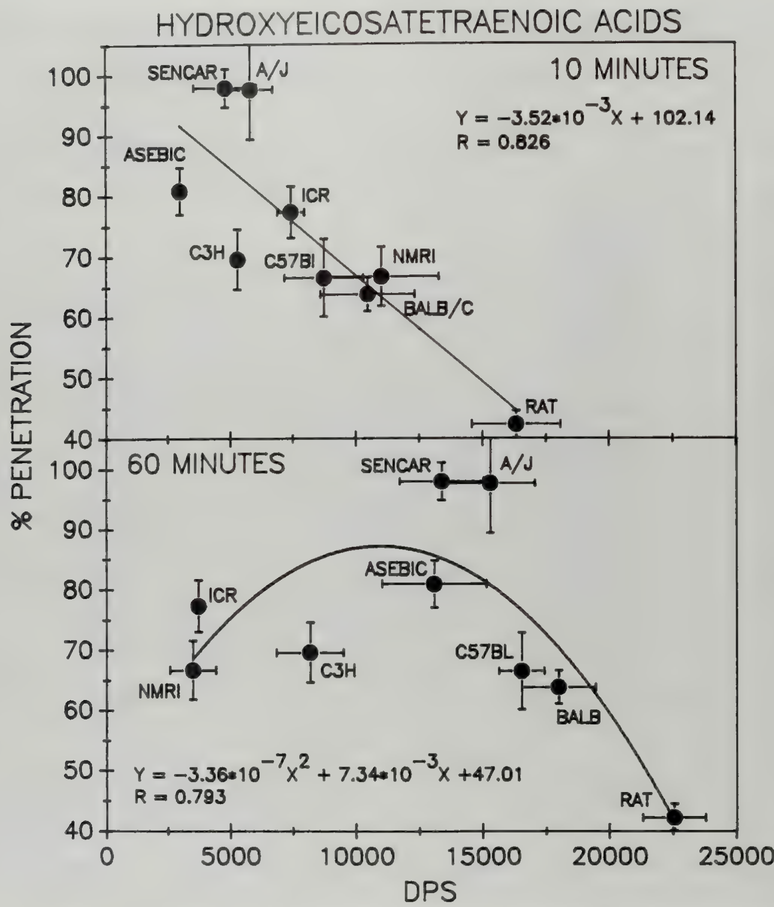

FIGURE 2. Tail skin HETE production as a correlate of cercarial penetration. Eicosanoid production was measured via HPLC as detailed in Materials and Methods. Total prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids were calculated by summing total DPS collected from 7–24 min, 24–48 min, and 48–70 min, respectively. Data for percent penetration are presented in Figure 1. Data for PG and LT production are not shown because no overall correlations were observed, although 6 of the 9 strains showed a linear relationship between LT production and cercarial penetration. Error bars represent standard errors based on 11–13 replications of penetration data, except for ASEBIC mice in which only 7 replications were done, and 5–6 replications (ASEBIC mice, 3 replications) of eicosanoid data.

relation than at 10 min. A linear relationship existed only if the ICR, NMRI, and C3H strains were excluded from the equation. However, a parabola-shaped relationship fitted the data best indicating that low or high HETE levels may be detrimental to cercarial penetration ($Y = -3.36 \cdot 10^{-7}X^2 + 7.34 \cdot 10^{-3}X + 47.01$, $r = 0.793$). HETE production at 30 min was intermediate between the 10- and 60-min time periods having a linear correlation (r) of only 0.644. Skin HETE production at 30 and 60 min, which are time periods when cercariae would have already penetrated skin, may be less important than at 10 min, a time period more reflective of the time course of cercarial penetration.

DISCUSSION

The data reported here show that a relationship exists between the innate ability of tail skin to synthesize HETE and the ability of cercariae to penetrate tail skin. Each strain of mouse had differing levels of HETE production and exhibited strain-specific cercarial penetration rates.

Thus, mouse strains do vary in their *primary* response to cercarial invasion. This is particularly important as the published data dealing with strain-specific responses to cercarial invasion have concentrated on adult worm recoveries and/or the secondary response, after mice have been exposed to various "immunization" procedures. Although adult worm recoveries are related to skin penetration events, they are also a product of skin, lung, and liver attrition rate. Therefore, they cannot be compared with the results obtained in the present study.

A number of authors have tried to correlate "protection" to the MHC locus (Colley and Freeman, 1983; Jones et al., 1983), Rsm-1 locus (Correa-Oliveira et al., 1986), macrophages (James et al., 1983, 1985; James and Cheever, 1985; James, 1986; James and DeBlois, 1986), and IgM (Correa-Oliveira et al., 1984) with variable success. Many of the studies demonstrating favorable correlations between resistance and an immunological parameter have looked at only 2 strains of mice (C57 and usually P or CBA). It is very clear that resistance to reinfection is a complex phenomenon depending not only on immunological mechanisms and the genetic background of the host, but also on as yet unidentified factors.

We believe part of the problem in understanding these reinfection experiments has been the lack of information on the primary infection process. Specifically, there has been a lack of good techniques to quantify cercarial penetration. Table I lists several authors who used various techniques for estimating skin penetration. In most cases the cut and mince technique was used, but Mastin et al. (1983) state that this technique underestimates the numbers of cercariae that penetrate the skin. On the other hand, counting cercariae remaining after exposure to tail or belly skin tends to overestimate the numbers of cercariae that have successfully penetrated. Histologically, determining the numbers of cercariae that penetrate may be a more accurate, yet tedious method. With the advent of the autoradiographic method of Georgi (1982), a highly reproducible and accurate method of measuring cercarial penetration is available to investigators. Table II lists a number of investigators using this technique to measure cercarial penetration rates. Unfortunately, none of these investigators examined strain-specific differences in cercarial *penetration*.

Even when a reliable method for measuring

TABLE I. Schistosoma mansoni: *Cercarial penetration rates (percent) using various techniques and strains of mice.*

	Authors						
	Smithers and Gammage, 1980	Miller and Smithers, 1982	Miller and Smithers, 1980	Fanning and Kazura, 1984	Stirewalt et al., 1965	Warren and Peters, 1967	Mastin et al., 1983
Measurement method	Cut and mince	Cut and mince	Cut and mince	Cut and mince	No. recovered*	No. recovered*	Histology
Site of application	Belly	Belly	Belly	Belly	Tails	Belly	Ear
Application time	10 min	10 min	10 min	10 min	1 hr	10 min	?
No. cercariae applied	112–141	107–166	136–158	~250	~50	~40	196–200
Time after application	24 hr	48 hr	48 hr	24 hr	N/A†	N/A	24 hr
Strains							
C3H	–	–	–	–	94	–	–
Swiss	–	–	–	–	93	88	–
Beige	–	–	–	–	87	–	–
Hairless	–	–	–	–	85	–	–
BALB/c	–	–	–	28	–	–	–
C57Bl/6	–	41	–	36	–	–	–
CBA	27	34	32	34	–	–	54

* The number of cercariae remaining was subtracted from the number applied. All others were assumed to have successfully penetrated.
† N/A = data not available.

TABLE II. Schistosoma mansoni: *Penetration of ^{75}Se-labeled cercariae (percent) in various strains of mice.*

	Authors					
	Present study	Dean et al., 1984	Mangold and Dean, 1983	Georgi et al., 1986	Dean and Mangold, 1984	Knopf et al., 1986
Site of application	Tail—1 hr	Tail—1 hr	Tail—1 hr	Belly—30 min	Tail—1 hr	Belly—30 min
No. cercariae applied	~75	~180	~50	135–231	168	~200
Time after application	24 hr	24 hr	24 hr	24 hr	24 hr	48 hr
Strains:						
C3H	69	–	–	–	–	–
BALB/c	63	–	–	–	–	–
C57Bl/6	66	77	85	–	–	–
CBA	–	–	–	27	–	–
SENCAR	97	–	–	–	–	–
NMRI	66	–	–	–	64 (80)*	–
A/J	97	–	–	–	–	–
ICR	77	–	–	–	–	–
CD-F rat	–	–	–	–	–	69 (79)†
SD-Rat	43	–	–	–	–	–

* 64% penetration for female mice 24 wk of age; 80% at 14 wk of age.
† 69% penetration for rats weighing 65–80 g; 79% for 70–90-g rats.

cercarial penetration is available, other problems remain. For instance, cercarial penetration rates may vary between tail, belly, and ear skin. The length of time cercariae are in contact with skin tissue may have an influence on cercarial penetration. Factors such as age of cercariae, batch of cercariae, temperature, volume of water in which cercariae are applied, housing conditions of mice, sex of the host, age of host, and whether the host was anesthetized may all contribute to the ability of cercariae to penetrate host skin. This paper demonstrates that the strain of mouse will also influence cercarial penetration rates. Thus, many investigators feel that cercarial penetration is so variable that accurate measurements are impossible or dubious at best.

We sought to minimize the problems described above by the following techniques: using cercariae under 2 hr old; exposing only tail skin; keeping the temperature between 21 and 23 C; using similarly aged mice (4–6 wk); keeping all mice under identical housing conditions on hardwood bedding; shedding cercariae at the same time daily; waiting 3 days between repeated snail sheds; and using the ICR strain as a type of internal control to monitor cercarial batches. In our hands, using the above protocol, the penetration rates within strains were consistent and we include the standard error bars in Figure 1 for comparisons, though we do acknowledge that varying from the above protocols will give differing penetration rates in a given strain. For instance, repeatedly shedding snails every 12–24 hr has given us penetration rates [illegible] as 35% in ICR mice; thus, the importance of following a rigid infection protocol is emphasized.

Table II lists cercarial penetration rates for other investigators using the ^{75}Se technique; unfortunately, only 2 strains can be compared: C57Bl/6 and NMRI. Our results for C57Bl/6 mice are 10–20% lower than those obtained by Dean et al. (1984) and Mangold and Dean (1983). The differences between means may be due to the fact that we used male mice 4–6 wk of age whereas Dean used female mice 8–9 wk of age. In addition, the housing conditions of the mice were not noted in either paper. In comparing our data for percent penetration in the NMRI strain with data obtained by Dean and Mangold (1984), we find, once again, that the age and sex of the host do not match. Dean and Mangold used female mice at 14 and 24 wk of age whereas we used male mice 4–6 wk of age. Clearly studies need to be undertaken examining ^{75}Se-labeled cercarial penetration rates and host age, sex, and strain parameters under rigid experimental conditions. Given the reasons discussed above, we do not believe that data obtained with ^{75}Se-labeled cercariae can be compared with data obtained by the chop and mince, percent remaining, or adult worm recovery techniques.

We have determined that mice vary in their response to cercarial infection via the tail route. On the other hand, a major correlation for cercarial resistance in primary infections is overall skin HETE production. Correlations between overall PG and LT levels, cercarial penetration, and mouse strain were not noted. This is not

surprising given the wide differences of biological activities that each of these eicosanoid classes possess. Although a correlation between overall skin HETE production and cercarial skin penetration exists, this does not rule out other factors that may be important. We believe that successful cercarial penetration is a correlate of many variables, not just overall skin HETE production. Indeed, our data imply that other factors may be important because several strains appear to vary from the linear relationship noted in Figure 2. In fact, we have obtained excellent linear regression lines between LT production, cercarial penetration, and mouse strain if 3 strains, ⅓ of the strains studied, were eliminated from the regression equation. This fact strongly suggests that skin LT levels may play some role in penetration for many strains of mice, but that there are other factors (particularly seen in the A/J, SENCAR, and SD-Rat strains) that override LT production resulting in a poor overall correlation coefficient. Other factors that may be involved in cercarial skin penetration are skin surface essential fatty acid levels, epidermal or dermal eicosanoid production, individual skin HETE species or interactions between them, cercarial production of eicosanoids, as well as the various immunological factors discussed above.

Skin surface fatty acid levels may be important because cercariae are stimulated to penetrate (*in vitro*) by essential fatty acids, which are also precursors for eicosanoid production (Salafsky et al., 1984a, 1984b). Thus, decreased skin levels of essential fatty acids or increased levels of skin nonessential fatty acids (nonstimulants) may decrease cercarial stimulation and subsequent penetration. We would also expect increased skin essential fatty acids or decreased skin nonessential fatty acids to increase cercarial stimulation and penetration. In order to test the role of sebum excretion on cercarial penetration rates, the ASEBIC mouse strain was included in this study. Given the high penetration rate (80%) of this strain, skin sebum excretion does not appear to be a factor influencing cercarial penetration; however, skin lipid composition (especially free fatty acid) may still be important. Several other excretion mechanisms are available to deposit fatty acids on the skin surface.

The quantities and types of epidermal or dermal eicosanoids released may also be important. Because we examined dermal and epidermal eicosanoid production taken together, we cannot evaluate which tissue stratum has the major role in responding to cercarial penetration, though we believe the epidermal tissue is the prime candidate because cercariae initially penetrate this tissue and remain in it for up to 24–48 hr before entering the dermal layer (Wheater and Wilson, 1979; Incani and McLaren, 1984). Purified mouse epidermal keratinocytes are known to produce mainly 12-HETE, 15-HETE, PGE_2, PGF_{2A} (Fortune et al., 1985), and 5-HETE and LTB_4 (Ziboh, 1985). Mouse epidermal homogenates produce 12-HETE, PGE_2, and PGF_{2A} (Bedford et al., 1983). In addition, the data reported here point to individual strain variations in quantity and type of tail skin eicosanoids produced by mice. We also note that we have only measured total HETE production. Variations between strains and within time may also occur regarding individual HETE species.

Cercariae also produce their own eicosanoids when stimulated by essential fatty acids (Fusco et al., 1985). An interplay between cercarial production and skin production may also be important in restricting or enhancing cercarial penetration. We have previously reported that cercarial LT and HETE production are highly correlated to penetration (Fusco et al., 1986). In addition, the present data correlate skin HETE production at both 10 and 60 min with cercarial penetration. These data lend support to the proposal that the interaction between cercarial and skin eicosanoid production modulates penetration. Indeed such an interaction has already been shown between dermal and epidermal tissue. Kragballe et al. (1986) have shown that dermal production of 15-HETE actually inhibits epidermal 12-lipoxygenase products such as 12-HETE. Such an interaction between cercarial and skin eicosanoid production may occur because certain HETE species are strong chemoattractants for various inflammatory cells (Goetzl et al., 1980a, 1980b). Increasing levels of these HETE species would be expected to reduce penetration, and indeed this is seen at 10 min and, to a lesser extent, at 60 min.

Several questions now need to be resolved: (1) How does cercarial eicosanoid production modulate skin eicosanoids and therefore enhance (or reduce) penetration levels? (2) What cell or tissue types are involved in producing skin eicosanoid in response to cercarial invasion? (3) Can modulation of skin eicosanoid production be used to control the infection process? (4) Can eicosanoid levels in secondary infections be correlated to resistance to reinfection?

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IN VIVO AND *IN VITRO* EGG PRODUCTION BY *NEMATOSPIROIDES DUBIUS* DURING PRIMARY AND CHALLENGE INFECTIONS IN RESISTANT AND SUSCEPTIBLE STRAINS OF MICE

F. Javier Enriquez*, Virginia Scarpino, Raymond H. Cypess, and Donald L. Wassom†

Department of Microbiology, Immunology, and Parasitology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

ABSTRACT: Experiments were conducted to examine adult worm burdens, fecal egg output, and *in vitro* fecundity of *Nematospiroides dubius* in resistant LAF1 and susceptible CBA mice 12, 15, 18, and 21 days following primary and challenge infections. A strong correlation was obtained on the number of eggs produced by worms cultured *in vitro* and the egg production as assessed by fecal egg count. Worm counts, fecal egg counts, and *in vitro* fecundity were similar on all days studied following a primary infection in both mouse strains. However, after challenge infection, LAF1 mice showed lower worm burdens, fecal egg output, and *in vitro* egg production when compared to CBA mice. Although the egg production of surviving female worms from immune LAF1 mice was decreased, it never fell below a threshold of 100 eggs/day. The reduced fecundity may be a manifestation of a general anti-worm response rather than responses directed specifically at worm reproduction.

Virtually all strains of mice are susceptible to a primary infection with *Nematospiroides dubius,* but strains may differ markedly in their ability to resist a challenge infection (Jacobson et al., 1982). Although it is not known how the anti-parasite response is effected in resistant hosts, a number of possible targets for this response have been proposed: decreased penetration of the infective larvae into tissues of the gastrointestinal tract (Panter, 1969b); retardation of development of larval tissue stages (Barlett and Ball, 1974; Behnke and Parish, 1979b; Ey et al., 1981); host killing of larvae within the intestinal mucosa (Barlett and Ball, 1974; Chaicumpa et al., 1977); decreased fecundity (Kerboeuf, 1982; Kerboeuf and Jovilet, 1984); and expulsion of adult worms (Panter, 1969a; Barlett and Ball, 1974; Prowse et al., 1979).

It is known that fecal egg output is decreased in mice receiving challenge infections when compared with primary infection controls (Kerboeuf, 1985). However, it is not known if this reduction in egg output is due to loss of adult worms from these mice and/or if adult female worms, which establish in resistant hosts, also produce fewer eggs. To address this question, we harvested adult female worms from the small intestine of resistant and susceptible mice (Jacobson et al., 1982) and counted the number of eggs produced by these worms *in vitro* following incubation in medium designed to assess levels of newborn larval production by *Trichinella spiralis* female worms (Dennis et al., 1970). Results were compared with the fecal output of similarly infected mice as determined by the method of Brindley and Dobson (1982).

Our results show a strong correlation between the *in vivo* and the *in vitro* assay systems, and demonstrate that, following a challenge infection, female worms taken from resistant mice are less fecund than worms taken from susceptible mice.

MATERIALS AND METHODS

Mice

Eight-week-old female LAF1 and CBA mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Parasite

Methods for maintaining *N. dubius* in the laboratory and for infecting experimental mice were exactly as described previously (Jacobson et al., 1982).

Anthelminthic

Ivermectin (Eqvalan MSD Agvet Inc., Barceloneta, Puerto Rico) was administered per os (8 mg/kg body weight) with an 18-gauge blunt-curve needle attached to a 1-ml syringe.

Fecundity assays

Fecundity of worms *in vivo* was determined according to the method of Brindley and Dobson (1982) and

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* Present address and correspondence: Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721.

† Present address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, Wisconsin 53706.

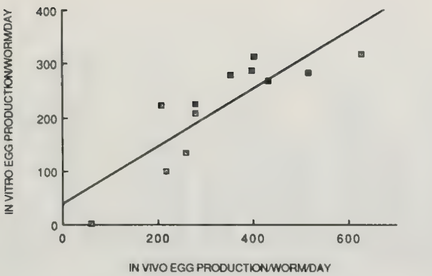

FIGURE 1. Comparison of the *in vivo* and *in vitro* *N. dubius* fecundity assay after primary and secondary infections of LAF1 mice. Each point indicates the mean EPFWD (calculated *in vivo* from fecal egg counts and the *in vitro* fecundity assay), from a group of 6 mice.

results are expressed as eggs per female worm per day (EPFWD). Between the hours of 0900 and 1100 on the day of necropsy each mouse was weighed and placed individually into a cage containing moistened paper towels for exactly 1 hr. Fecal material was collected from the cages and weighed, and the eggs per gram of feces were determined using quantitative chambers (Hawksley, England) and the technique of McMaster (Brindley and Dobson, 1981). Fecundity was also estimated using an *in vitro* assay. Female worms were removed from the small intestine, washed 5× at 1 *g* in phosphate-buffered saline (pH 7.2) containing 100 IU penicillin and 100 μg streptomycin/ml (GIBCO, Chagrin Falls, Ohio), and at least 10 female worms per mouse were placed individually into wells of sterile 96-well microtiter plates (Costar, Cambridge, Massachusetts) containing 0.2 ml of RPMI-1640 (GIBCO) supplemented with 29% fetal bovine serum (GIBCO), and 1% penicillin–streptomycin solution (GIBCO). After 24 hr incubation at 37 C, 5% CO_2, eggs produced by each worm were counted at 20× using a Biostar inverted microscope.

Experimental design

The daily fecal output was calculated from the regression Y (g feces/hr) = 0.129 + 0.00533(mouse body weight) for CBA mice and Y = −0.040 + 0.0158(mouse body weight) for LAF1 mice. In order to assure the validity of using mouse weight as a predictor of grams of feces per day according to the method of Brindley and Dobson (1982), we performed a regression analysis comparing live mouse body weight and the grams of fecal material voided per hour.

To determine if the *in vitro* egg production assay was predictive of values obtained from fecal egg counts, we infected CBA mice with 50 infective third-stage larvae (L3) of *N. dubius*, treated the mice with ivermectin on day 14, and on day 21 reinfected them along with an equal number of primary infection controls with 100 L3 of *N. dubius*. Values of EPFWD obtained for CBA mice using the *in vivo* and *in vitro* method were compared on days 12, 15, and 18 postinfection.

For experiments shown in Figures 3, 4, and 5, LAF1/J and CBA/J mice were divided into 8 groups of 6 mice each. Groups 5–8 were infected with 50 L3 of *N. dubius* on day 0; these mice along with mice in groups 1–4

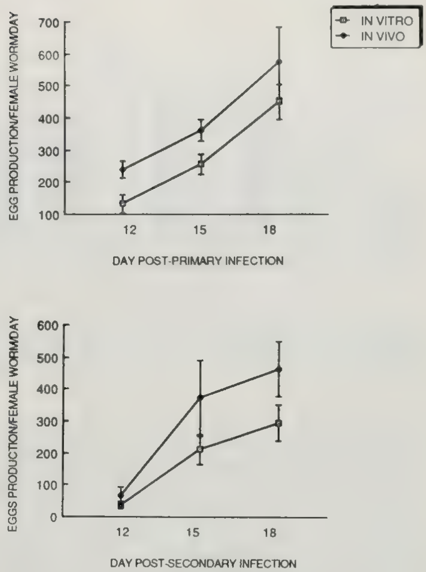

FIGURE 2. Kinetics of egg production by *N. dubius* as assessed by the *in vivo* and *in vitro* assays on days 12, 15, and 18 after primary and secondary *N. dubius* infections of CBA mice.

were treated with ivermectin (8 mg/kg) on day 14, and on day 21, all mice were infected with 100 L3. Thus, mice in groups 1–4 served as primary infection controls for mice in groups 5–8, which were primed with 50 L3 prior to challenge. Groups 1 and 5 were sacrificed for worm counts and *in vitro* assays on day 33, groups 2 and 6 were killed on day 36, groups 3 and 7 on day 39, and groups 4 and 8 were sacrificed on day 42 (days 12, 15, 18, and 21 postinfection/challenge, respectively). At necropsy, worms were counted and females removed for washing and incubation in fecundity medium as described above. Worm counts, fecal egg outputs, and *in vitro* egg production on each of days 12, 15, 18, and 21 postinfection/challenge were compared between the different groups of mice.

Analysis of results

Significance was determined by the nonparametric Mann-Whitney Rank Sum Test and by simple linear regression (Snedecor and Cochran, 1980).

RESULTS

A positive correlation (r = 0.89, df: 47 for CBA, 46 for LAF1) was obtained for the daily fecal output and the weight of individual mice.

The *in vitro* egg production assay was predictive of values obtained from fecal egg counts. Although estimates of egg counts were higher using the *in vivo* method, there was a strong correlation (r = 0.89) (Fig. 1) between the results

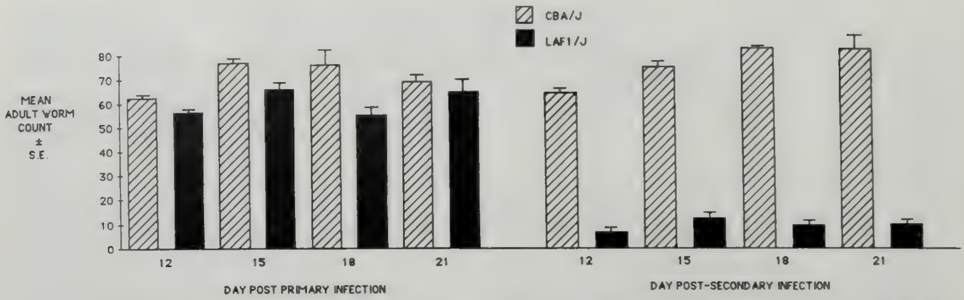

FIGURE 3. Number of adult worms recovered on days 12, 15, 18, and 21 post-primary and post-secondary infection with *N. dubius* in LAF1 and CBA mice. Mice were infected per os with 100 L3 larvae in both primary and secondary infections.

obtained using the *in vivo* and *in vitro* methods (Fig. 2a, b).

In an experiment of similar design, we compared the adult worm burdens and the egg production *in vivo* and *in vitro* on days 12, 15, 18, and 21 postinfection/challenge in CBA and LFA1. Results are shown in Figures 3–5. Worm counts for LAF1 and CBA mice were similar following a primary infection, but only LAF1 mice resisted the secondary challenge (Fig. 3). Likewise, although LAF1 mice passed slightly fewer eggs per gram of feces than did CBA mice during a primary infection, egg output of LAF1 mice was significantly lower ($P < 0.01$) following challenge. In contrast, fecal egg output of CBA mice did not differ between challenged mice and primary infection controls ($P > 0.05$) (Fig. 4).

The number of eggs produced *in vitro* by female worms harvested from mice in the above studies paralleled the results obtained from counting eggs in feces (Fig. 5). In addition, on days 12, 15, 18, and 21 postchallenge, female worms remaining in resistant LAF1 mice produced significantly fewer eggs *in vitro* than did worms harvested from primary infection controls ($P < 0.01$). Therefore, although worms may establish and reproduce in resistant mice challenged with *N. dubius,* such worms are less fecund than worms from primary infection controls, or from hosts that fail to develop resistance to challenge ($P > 0.05$).

DISCUSSION

Although the estimates of egg production per worm tend to be lower for the *in vitro* assay than for the assay wherein fecal egg counts were performed, there is an excellent correlation between the 2 assays in terms of the patterns of egg production observed. Thus, using either method, egg production tends to increase quickly following emergence of adult worms into the gut lumen, and fecal egg production reflects the numbers of adult worms present in the host (Behnke and Parish, 1979a; Sitepu and Dobson, 1982). The *in vitro* assay, however, is convenient to perform and allows one to estimate the variability in egg

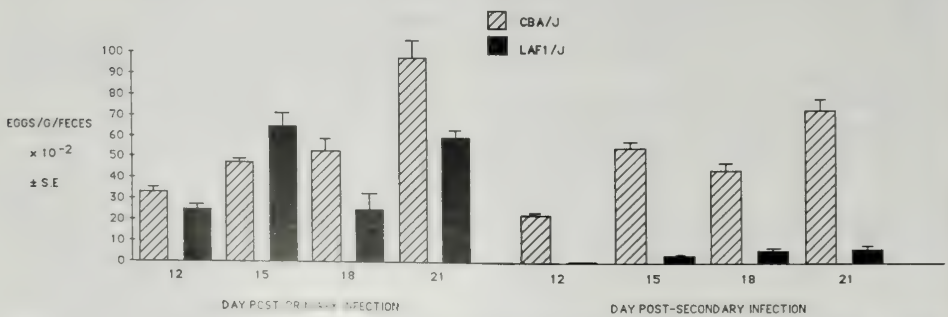

FIGURE 4. Fecal egg counts 12, 15, 18, and 21 days following either a primary or a secondary *N. dubius* infection in LAF1 and CBA mice. Mice were inoculated with 100 L3 in both primary and secondary infections.

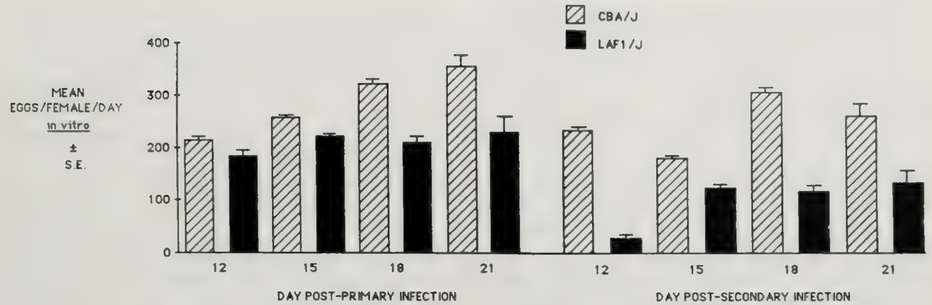

FIGURE 5. *In vitro* fecundity assay of *N. dubius* recovered at days 12, 15, 18, and 21 post-primary and post-secondary infection in resistant LAF1 and susceptible CBA mice. Mice were inoculated with 100 L3 larvae in both primary and secondary infections.

production among different worms in the infection population. We have repeated the experiments described above using a 6-hr rather than a 24-hr *in vitro* incubation and found that estimates of egg production per female worm per day are higher when the shorter incubation time is used. This suggests that the worms cultured under these *in vitro* conditions are releasing eggs at a progressively slower rate the longer they are kept in culture (data not shown). The shorter incubation times, therefore, may provide more accurate estimates of actual egg output than would the longer incubations and still would provide enough eggs for statistical analysis. Thus, there is no need to culture worms for longer than 5–6 hr, at which time the plates can be frozen and stored for counting at the investigators convenience. The principal advantage of the *in vitro* assay is that changes in host fecal volume, relative to diet or other non-worm-related factors, will not markedly affect the results observed.

As previously reported (Jacobson et al., 1982), LAF1 mice resisted challenge infection when compared with CBA mice. In addition we have observed that fecal egg output is markedly reduced in LAF1 mice that receive a challenge infection and the reduced fecal egg counts are attributable to at least 2 factors: (1) fewer worms established in the gut, and (2) reduced fecundity of established worms. We suspect that the reduced fecundity of female worms is a manifestation of more general anti-worm immune responses rather than responses directed specifically at worm reproduction. However, further studies will be required to determine if different anti-worm and stage-specific effects are related to distinct or common immunological effectors. Interestingly, female worms taken from highly resistant hosts were still able to reproduce, albeit at a slower rate, and egg production never dropped below a threshold of 100 eggs per female per day (Kerboeuf and Jovilet, 1984). This would suggest that an immune response directed specifically at worm reproduction, if it occurs at all, is not particularly effective. Nonetheless, our results demonstrate that egg counts, whether performed following the *in vitro* culture of female worms, or whether performed on measured samples of voided feces, accurately reflect the immune status of the host and may be used as an adjunct to other available methods in assessing levels of resistance to *N. dubius* infections of mice.

ACKNOWLEDGMENT

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A NEW METHOD FOR CLONING *GIARDIA LAMBLIA*, WITH A DISCUSSION OF THE STATISTICAL CONSIDERATIONS OF LIMITING DILUTION

K. F. Baum, R. L. Berens, R. H. Jones*, and J. J. Marr

The Division of Infectious Diseases, The Department of Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Street, Denver, Colorado 80262

ABSTRACT: This report describes a method of cloning *Giardia lamblia* by limiting dilution which is simpler than the previously described semisolid agar technique and which may also be applied as an assay of cell viability. A discussion of the basic statistics of limiting dilution, which is applicable to any cell type, and a method of statistically comparing colony-forming efficiencies from different cell populations are included. The colony-forming efficiency (CFE) of this method, when applied to late log-phase cultures, is 72.1 ± 10.05%. When only cells adherent to the sides of culture vials are cloned, the CFE is 87.1 ± 9.85%.

The axenic culture of *Giardia lamblia* trophozoites, first reported by Meyer (1976), enabled biochemical and metabolic investigations that had previously been impossible. Cloning of the organism allows further precision in these investigations by ensuring genetic homogeneity of the cell population. It is an especially important tool in the analysis of naturally occurring strain differences and laboratory-selected mutants. The growth of colonies from single cells also permits qualitative assessments of cell viability. A method of cloning in semisolid agar with a CFE (colony-forming efficiency) of 20–40% has been reported (Gillin and Diamond, 1980). The present report describes a cloning method with an improved CFE using the technically simpler method of limiting dilution.

MATERIALS AND METHODS

The strains of *Giardia* that were used include WB (ATCC 30957) as well as mutants of this strain developed in this laboratory. The mutants were cells resistant to purine nucleoside analogs obtained by standard somatic cell genetic techniques. Cells were grown in Diamond's filter-sterilized TYI-S-33 medium as modified by Keister (Keister, 1983) with the following further modifications: trypticase replaced with casein digest (BBL, Cockeysville, Maryland), ferric ammonium citrate deleted, 50 mg dehydrated bovine bile/100 cc, fetal calf serum was not inactivated, 3 ml of Diamond's Tween-80 solution/100 cc was added, and the pH was adjusted to 6.86. A starting concentration of 10^4 cells/ml was grown in 72 hr to late log phase (approximately 10^6 cells/ml) in 13 × 100-mm borosilicate screw-top culture tubes. The tubes were inverted twice and the culture medium was decanted and replaced with fresh, cold medium. Tubes were placed in an ice bath for an additional 15 min. With this method, only cells attached to the culture tube wall, presumably the most viable, were used for cloning. To compare CFE, other tubes were chilled without decanting nonadherent cells. The cells were counted in a hemocytometer and serially diluted in cold medium to the desired concentration; e.g., 0.26 cells/ml, which gives approximately 5 predicted colony-forming units (CFU) per 19.2 ml, the capacity of a 96-well plate (0.26 cells/ml in 19.2 ml yields 5 cells). The dilute cell suspension was mixed and 0.2-ml aliquots were transferred into each well of a sterile flat-bottom 96-well microtiter plate (Corning Glass Works, Corning, New York). The plate was covered and placed in a plastic bag with a water-soaked gauze pad to prevent evaporation of culture medium. This was placed in an anaerobic incubator (Forma Model 1024, Forma Scientific, Marietta, Ohio) with a gas mixture of 90/9.5/0.5 $N_2/CO_2/O_2$. After 30 min equilibration, the plate was sealed in the bag with a wire twist-tie and incubated for 4–6 days at 37 C. Cell growth in a well was readily apparent with an inverted phase microscope at 100–200× magnification. Organisms were retrieved by placing the plate in ice water for 15 min, mixing each well several times with a sterile-tipped Eppendorf pipette apparatus, and inoculating the contents of the well into a 13 × 100-mm culture tube containing fresh medium.

The statistical analysis of limiting dilution is based on the following formulas, which incorporate the fact that cells are inoculated into the plate in a Poisson distribution (Lefkovits and Waldman, 1979). The most important information in cloning is the colony-forming efficiency (CFE) and the certainty of a positive well representing a true clone. To determine these, the values for the predicted number of positive wells (assuming 100% CFE, and knowing the mean inoculum as determined by dilution) and the predicted number of singly occupied wells must be determined.

If an average of C cells is randomly distributed among W wells, the average number of cells per well is C/W.

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* The Department of Preventive Medicine and Biometrics, University of Colorado Health Sciences Center, 4200 East Ninth Street, Denver, Colorado 80262

Incorporating the Poisson distribution, the probability of any given well being singly occupied is

$$(C/W)(e^{-C/W}),$$

and the expected number of singly occupied wells is

$$C(e^{-C/W}).$$

The expected number of occupied wells is

$$W(1 - e^{-C/W}),$$

so the certainty of a single well being a true clone is

$$(C/W)e^{-C/W}/(1 - e^{-C/W}).$$

CFE, best determined with a higher inoculum (e.g., 100 cells in 96 wells), is determined by the number of observed positive wells divided by the mean number of predicted positive wells multiplied by 100, i.e., O/P × 100 (maximum value 100%). A variation of the usual chi-square test can be used to test whether 2 or more CFE's are the same. Let λ_i be the predicted number of positive wells for experiment i, n_i be the actual number observed, and e_i be the predicted efficiency (calculated below). Arrange the data in a table:

	Experiment				
	1	2	...	m	Total
λ	λ_1	λ_2	...	λ_m	λ_t
n	n_1	n_2	...	n_m	n_t
e	e_1	e_2	...	e_m	

The CFE if both experiments are the same is estimated from the total column:

$$CFE = n_t/\lambda_t.$$

If this efficiency is the same for both experiments, the expected number of positive wells for experiment i is

$$e_i = \lambda_i CFE = \lambda_i n_t/\lambda_t.$$

The chi-square test is calculated in the usual way:

$$\chi^2_{m-1} = \Sigma\,(n_i - e_i)^2/e_i.$$

A value of χ^2_{m-1} exceeding the value from a table for m − 1 degrees of freedom rejects the hypothesis that the CFE's for all the experiments are the same. Below is an example:

	Experiment		
	1	2	Total
λ	63.43	62.37	125.8
n	50	61	111
e	55.97	55.03	

The CFE estimated from the data pooled over the 2 experiments is 88.24%. The chi-square value is

$$\chi^2 = (50 - 55.97)^2/55.97 + (61 - 55.03)^2/55.03 = 1.28$$

and the hypothesis that the CFE for the 2 experiments is the same is not rejected.

RESULTS

The overall CFE when initial cell cultures are not decanted prior to placing in an ice bath is 72.1 ± 10.05% (Table I). The CFE of cultures that have been decanted and refilled with fresh medium before icing and diluting is 87.1 ± 9.85% (Table II). The difference in CFE is not significant. There were no significant differences between the CFE's of any of the strains cloned in the decanted or nondecanted vial experiments, but there was a trend toward a higher CFE in the wild-type WB strain (95.8 ± 6.63%) in comparison to that observed in a mutant strain resistant to multiple purine nucleosides (76.2 ± 5.39%).

DISCUSSION

Two factors reduce the probability of a single positive well representing a true clone. First, more than 1 organism may occasionally be inoculated into 1 well. The chance of this occurring with a

TABLE I *Results of limiting dilution experiments using undecanted vials. CFE = 72.1 ± 10.05 (SD)%.*

Mean no. organisms inoculated	No. of wells	Predicted positive wells	Actual positive wells
2.0	96	1.98	3
4.8	96	4.68	2
4.8	96	4.68	2
4.8	96	4.68	3
5.61	95	5.47	2
5.8	96	5.59	6
7.2	96	6.93	3
7.3	95	7.05	8
9.6	96	9.13	9
16.8	84	15.22	15
19.8	95	17.85	9
20.0	96	18.04	9
20.0	96	18.04	12
20.0	96	18.04	16
Total:		137.38	99

TABLE II. *Results of limiting dilution experiments using decanted vials. CFE = 87.1 ± 9.85 (SD)%.*

Mean no. orgamisms inoculated	No. of wells	Predicted positive wells	Actual positive wells
2.0	96	1.98	2
2.0	96	1.98	3
4.9	95	4.73	5
5.0	96	4.87	5
5.0	96	4.87	5
9.6	94	9.11	11
10.0	96	9.49	7
10.0	96	9.49	4
19.8	95	17.85	22
19.8	95	17.85	12
20.0	96	18.04	15
20.0	96	18.04	12
Total:		118.30	103

small inoculum (e.g., 2 cells per 96-well plate) is minimal (1%). A second factor reducing the probability of a single well representing a true clone is the tendency for *Giardia* to appear as clumps in culture. When cultures have been decanted before chilling, 2.2% of all potential CFU (i.e., single organisms and clumps) in a late log-phase culture exist as multiples of organisms (excluding cells obviously dividing); in undecanted, chilled vials 4.6% of all potential CFU exist as multiples of organisms (unpubl. data). Therefore, decanting initial cultures and diluting the organisms to 2 per 96-well plate would yield positive wells, each of which has a 96.8% certainty of being a true clone ($100 - [1.0 + 2.2]$%; summing these 2 probabilities of a well not representing a true clone is valid only when these probabilities are small). Repeating the cloning procedure once raises this level of certainty to 99.9%.

In addition to providing a means of cloning, this method can be modified to give an assay of cell viability by inoculating a higher number of organisms. For example, when 96 cells are inoculated per plate rather than 2 as in the previous example, the mean number of predicted positive wells is 60.9, and the percent viability is: [number of positive wells/60.9] $\times$ 100. Inoculations in this range allow for determination of a broad general range of CFE whereas inoculations of 500–1,000 cells per plate are more suited to determining 99% killing activity.

The CFE of the decanted and undecanted vials differed by 15%, with the decanted vials showing the higher CFE. This difference was not statistically significant, yet the results give an idea of the close comparability of the 2 populations of cells. The relative value of the 2 methods of harvesting cells is now more apparent and investigators can decide if this difference is sufficiently important in a study to justify decanting the approximately 60% of cells that are unattached in late log-phase cultures (data not shown).

There were no statistically significant differences in CFE among the strains tested, although a suggestive trend did exist in comparing normal WB strain *Giardia* with a strain resistant to multiple nucleosides. The CFE of the normal strain was 95.8 ± 6.63% and the mutant strain was 76.2 ± 5.39%. It is possible that by including mutant strains in our pooled analysis, we have underestimated the CFE of this technique under optimal conditions.

In summary, the highly efficient cloning technique described will not only facilitate the isolation of genetically homogeneous cell lines for metabolic and genetic investigations of *Giardia*, but also will allow an assessment of cell viability. Such an assessment can be done directly on a given cell culture or can serve as a standard in evaluating other indices of cell viability such as cell morphology, motility, or vital dye exclusion. The technique, with minor modifications, is also readily applicable to cloning other cell types. We have used it for cloning trypanosomes.

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IMMUNOCYTOLOCALIZATION STUDY OF THE EXTERNAL COVERING OF *TRICHINELLA SPIRALIS* MUSCLE LARVA

Yuzo Takahashi, Takako Uno, Toshimasa Nishiyama, Shoji Yamada, and Tsuneji Araki
Department of Parasitology, Nara Medical University, Kashihara Nara 634, Japan

ABSTRACT: The antibody-binding sites of the muscle larva of *Trichinella spiralis* were investigated by immunogold staining on the ultrathin sections of LR white resin. The antibodies, which were produced in the course of *T. spiralis* infection in rats, specifically bound to the inner layers of the body cuticle and the cuticle of the hindgut, but not to the cuticle of the esophagus. This is the first report that reveals the antigenic nature of the inner layers of the external coverings of *T. spiralis* larva.

The body cuticle of *Trichinella spiralis* is an extracellular material that covers the entire surface of the worm, and is the most obvious point of contact between the worm and the host. Because *T. spiralis* stimulates complex, stage-specific immunologic responses in the host (Philipp et al., 1980, 1981), antigenic presentation by the cuticle in a natural infection has attracted a great deal of attention (Silberstein, 1983). There is no doubt about the antigenicity of the cuticle surface judging from a number of the studies including those of Despommier et al. (1967), Gadea et al. (1967), Stankiewicz and Jeska (1973), Vernes et al. (1974), McLaren et al. (1977), Mackenzie et al. (1980), Philipp et al. (1980, 1981), Kim and Ledbetter (1981), and Silberstein and Despommier (1984). However, there are no immunocytochemical data available regarding the antigen in the cuticle inner layer. This lack of information may be due to technical limitations in immunocytochemical staining. Because the cuticle is considered to be impermeable to large molecules, antibodies for immunostaining may not penetrate deep inside the cuticle, i.e., the cuticle inner layer. The only way to expose the cuticle inner-layer antigen to the antibody is by thin-sectioning of the cuticle followed by immunostaining. This method allows the antibody access to the cuticle inner-layer antigen. However, because the antigen is found only in small amounts on the cut surface of the cuticle, immunofluorescence and immunoperoxidase light microscopic techniques are not sensitive enough to detect such a small amount of antigen.

This investigation deals with the localization of antigen in the cuticle of the muscle larva of *T. spiralis* taking advantage of recent methodological advances of postembedding immunostaining using a colloidal gold marker (Beesley, 1985).

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MATERIALS AND METHODS

Tissue preparation

Trichinella spiralis (Polish strain kindly supplied by Prof. T. Yamaguchi, University of Hirosaki, School of Medicine, Japan) was carried in ICR mice. Muscle larvae of more than 5 mo postinfection were isolated by pepsin-HCl digestion (Despommier, 1974), fixed in half-strength Karnovsky solution, dehydrated with ascending concentrations of alcohol, and embedded in LR white resin (London Resin Company Ltd., U.K.).

Antisera

Three Wistar rats were orally inoculated with infective larvae (8,000 parasites each) and bled after 36 wk. Antisera were separately frozen and stored until use.

Conjugation of protein A with colloidal gold

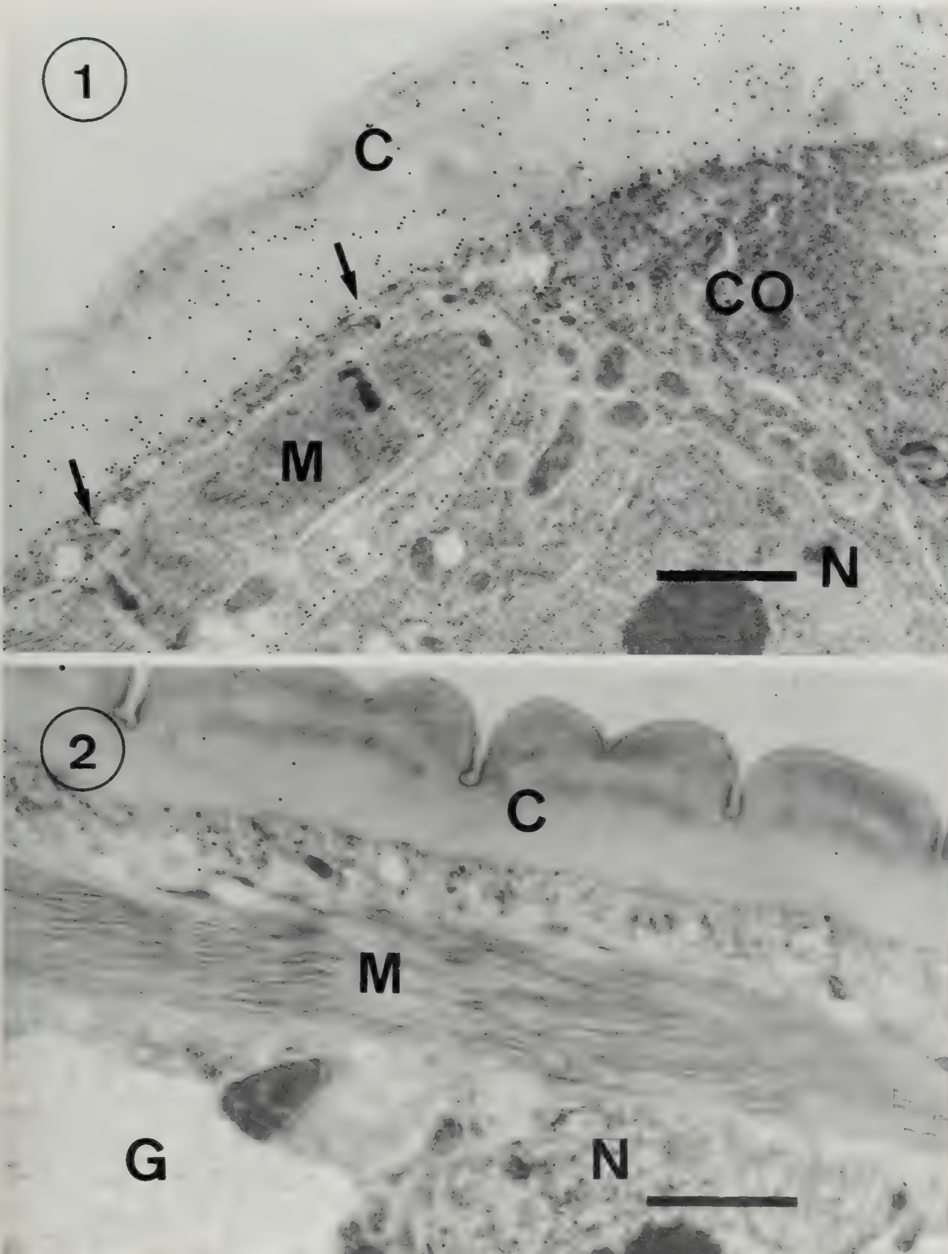
Colloidal gold particles, 15–17 nm diameter, were made by reducing chloroauric acid with trisodium citrate as previously described (Geoghegan and Ackerman, 1977). Protein A (Pharmacia Fine Chemicals Co.) was conjugated with the colloidal gold in the presence of 5% polyethyleneglycol (MW 20,000) and the conjugate was centrifuged at 55,000 *g* for 40 min on a 3-ml cushion of 5% glycerol containing 0.05% polyethylene glycol (MW = 20,000) and 0.01% sodium merthiolate. Condensed solution was recovered and 10-fold diluted with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (Tanaka et al., 1984).

Immunostaining

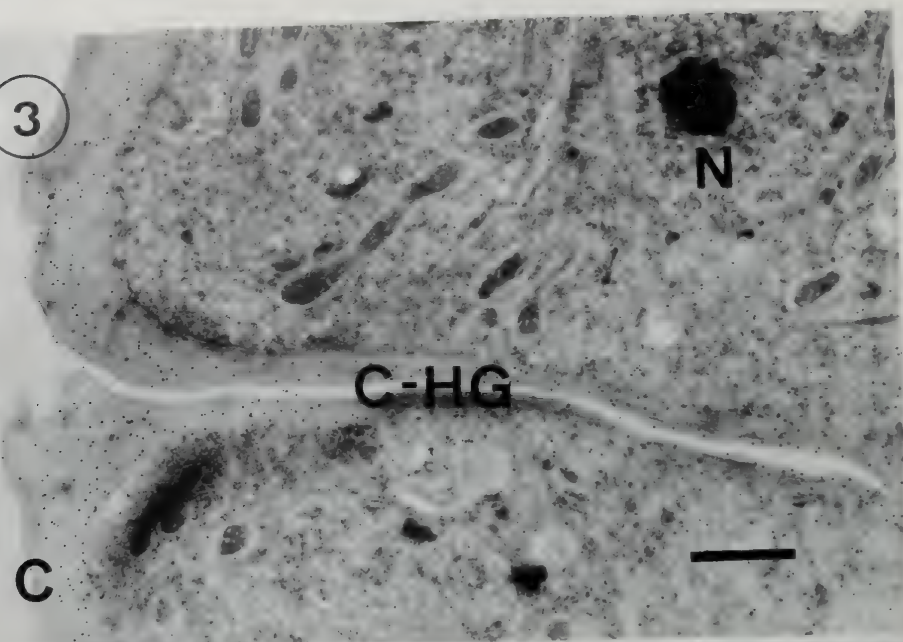
Immunostaining was performed on ultrathin sections of muscle larvae. Sections were incubated with infected and normal rat sera diluted 1:200 in PBS for 30 min at room temperature. After being washed 3 times, the sections were treated with protein A–gold conjugates for 30 min, washed with PBS, and stained with uranyl acetate.

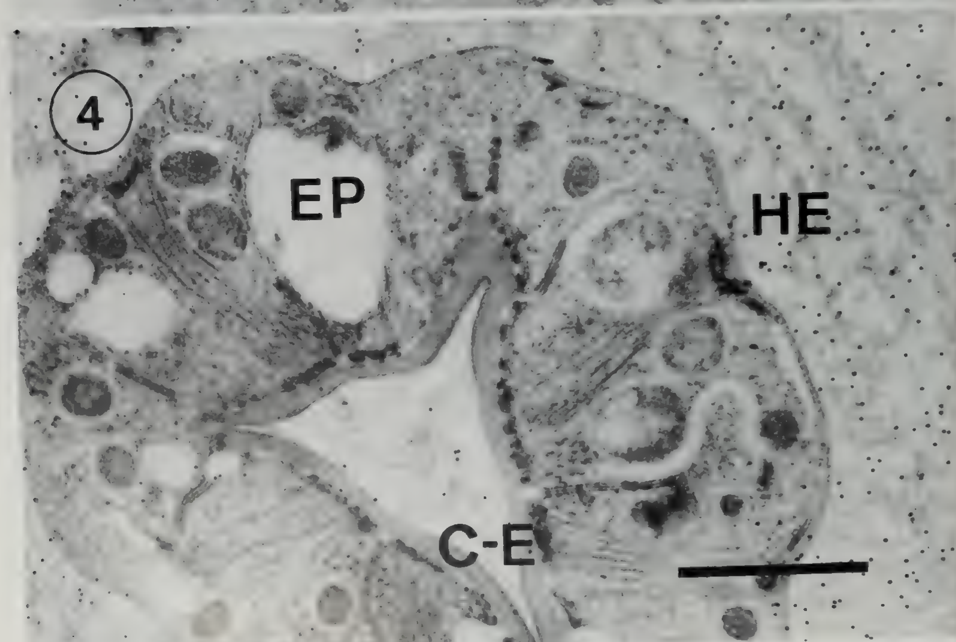
RESULTS

Figure 1 is a representative electron micrograph that shows the body cuticle of a muscle larva of *T. spiralis* stained with serum from the infected rat. Monomeric colloidal gold particles were uniformly distributed over the entire layer

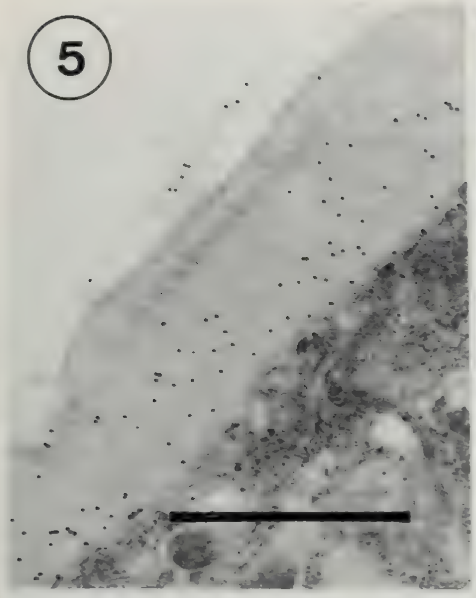


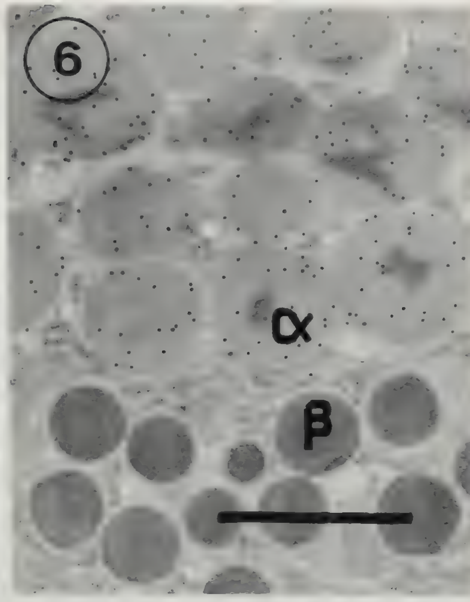
FIGURES 1, 2. **1.** Immunogold-stained larva that depicts dense and uniform staining on the cuticle inner layers (C). The thin extension of the cord (CO) is also positive (arrows). Nucleus (N) and myofibrils (M) are negative. **2.** No effective staining is observed in a control section. Glycogen (G). Bar = 1 μm.





FIGURES 3, 4. 3. A longitudinal section showing the cuticle lining of the hind gut (C-HG), which is continuous with the body cuticle (C). Both display the same positive staining pattern. **4.** A cross section of the esophagus. The cuticle (C-E) lining the epithelial cells (EP) is negative. The hemolymph (HE) is positive. Bar = 1 μm.





FIGURES 5, 6. **5.** Detection of surface antigen is possible when thick cuticle sloughs off. **6.** The used sera reacted with stichocyte α granules but not with β granules. Bar = 1 μm.

of the body cuticle (C) without regional preference. Background deposition on the nontissue area was minimal. The thin extension of the cord cell cytoplasm beneath the cuticle was also positive (an arrow in Fig. 1); however, the nucleus (N), mitochondria, and myofibrils (M) were always negative. Those organelles were a good built-in control.

Specificity of the staining was also confirmed by a control experiment. No effective staining was observed when sections were stained with normal rat sera (Fig. 2). Dense and uniform staining was shared by the cuticle lining the luminal surface of the hindgut (Fig. 3), but not by the cuticle lining the esophagus (Fig. 4). Our sera used also reacted with cuticle surface when the thick cuticle surface sloughed off (Fig. 5), and with stichocyte α granules, but not with stichocyte β granules (Fig. 6). Because the Wistar rat strain is not inbred, the staining pattern was carefully compared using sera from each of the 3 rats. The results were both consistent and reproducible.

DISCUSSION

The immunocytochemical staining method employed in these studies, namely postembedding using colloidal gold as a probe, seems to be a powerful tool for investigating antigens buried in solid tissue in such a way that in routine immunocytochemical studies, the antibody cannot have access to it. Although the number of antigenic moieties involved in immunoreaction on the cut surface of the sections is supposedly small, the immunogold technique was sensitive enough to detect such weak reaction as shown in Figures 1 and 3. The highly specific and reproducible results obtained led us to the conclusion that the body cuticle inner layers of the muscle larva of *T. spiralis* contains antigen(s) that is recognized by the host during infection. This seems to be the first report revealing the antigenic nature of the inner layers of the cuticle by means of immunocytochemical staining. The luminal surface of the esophagus and the hindgut is lined with an extracellular layer (external covering) that resembles the cuticle in appearance. Despite this morphological resemblance, our results show that the body cuticle and the external covering of the hindgut differ from that of the esophagus in antigen composition. Although in other species, some authors have already pointed out the fundamental differences between the body cuticle and the external covering of the esophagus, including reports by Kan and Davey (1968), Dick and Wright (1973), Wright (1976), and Vincent et al. (1975).

The antigen on the external covering of the esophagus was investigated by Despommier et al. (1967) at the electron microscopic level employing ferritin-conjugated antibody, underlining the absence of the antigen from the cuticle of the esophagus. The drawback of their method was that it did not eliminate the possibility that the absence of ferritin-conjugated antibody may simply have been due to the failure of the esophagus to be exposed to the antibody.

Unlike the cuticle inner layer antigen, the cuticle surface antigen was usually difficult to localize by the postembedding immunostaining. As shown in Figures 1 and 3, there was no preferential staining on the surface of the cuticle. However, detection of surface antigen was possible when thick cuticle surface sloughed off (Fig. 5), which suggests that cuticle surface is also antigenic.

The presence of antigen moieties in the inner layers of the cuticle was hereby demonstrated. However, the biological significance of this cuticle inner-layer antigen is thus far unknown. Taking advantage of the immuno-gold technique, our laboratory is currently studying the serological value of cuticle antigen, as well as stichocyte granule antigen, in immunodiagnosis by reacting the antigens with a panel of sera taken from patients with trichinosis, trichuriasis, clonorchiasis, filariasis, anisakiasis, and gnathostomiasis. Preliminary data showed that the cuticle inner-layer antigen had less specificity than the stichocyte granule antigen and can be used for screening purposes.

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ACTIVE AND PASSIVE IMMUNIZATION OF MICE AGAINST LARVAL *DIROFILARIA IMMITIS*

David Abraham*, Robert B. Grieve, Marcia Mika-Grieve, and Barbara P. Seibert†

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, Wisconsin 53706

ABSTRACT: The objective of this study was to determine if *Dirofilaria immitis* larvae would survive in diffusion chambers implanted in dogs and mice and secondly to determine if mice could be immunized against infection with *D. immitis. Dirofilaria immitis* third-stage larvae (L3) survived and grew in diffusion chambers implanted in dogs and mice for at least 3 wk. BALB/c mice, which were repeatedly infected with live L3, showed resistance to challenge infections. Dead L3, with or without adjuvants elicited no protective immunity. A correlation was found between the degree of immune protection seen in mice and antibody levels to soluble larval antigen but not to antibody levels to surface antigens. A monoclonal antibody was prepared that reacted with the surface of *D. immitis* and *Onchocerca lienalis* L3, but not to the surfaces of other stages and species of various filarial worms. When this antibody was administered to mice prior to challenge no significant reduction in larval survival was observed.

Research on immunity to filarial infections has been hindered by a paucity of host–parasite systems suitable for experimental manipulation in the laboratory (Philipp et al., 1984). Adult and microfilarial stages of *Dirofilaria immitis* have been successfully maintained in rodent hosts (Grieve and Lauria, 1983; Grieve et al., 1985) and the initial objective of the present study was to determine whether third-stage larvae (L3) and fourth-stage larvae (L4) of *D. immitis* could be maintained in mice. As a means of efficiently recovering larval stages of *D. immitis* from mice, diffusion chambers were employed. Diffusion chambers have been utilized in the study of the biology and immunology of a number of parasites, including *Schistosoma mansoni* (Kassis et al., 1979), *Trichostrongylus colubriformis* (Rothwell and Love, 1974), *Dipetalonema viteae* (Gass et al., 1979; Weiss and Tanner, 1979; Tanner and Weiss, 1981a; Abraham et al., 1986), *Brugia pahangi* (Court, 1982; Chandrashekar et al., 1985), *Onchocerca volvulus* (Strote, 1985), and *D. immitis* (Kobayakawa et al., 1974, 1976; Kobayakawa, 1975; Delves and Howells, 1985).

The second phase of this study utilized the *D. immitis*–mouse model for study of acquired protective immune responses to larval *D. immitis*. Mice were actively immunized with larvae or passively immunized with monoclonal antibody directed against an L3 surface antigen. The objectives of this segment of the study were to determine if mice could be immunized against infection with *D. immitis*, if antibody to L3 surface could confer protection, and to begin evaluation of the effector mechanisms associated with protective immunity.

MATERIALS AND METHODS

Recovery of L3

Aedes aegypti Liverpool (Blackeye strain) were infected with *D. immitis* by feeding on microfilaremic canine blood through an artificial membrane feeding apparatus (Rutledge et al., 1964). Fifteen days after infection, mosquitoes were anesthetized and surface-sterilized by immersion in 95% ethanol followed by a 3-min wash in 10% benzalkonium chloride in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) (Lok et al., 1984). Mosquitoes were washed 3 times in PBS and placed on 60-mesh screens inside funnels filled with tissue culture medium. Larvae were collected from the funnels 90 min postincubation.

Implantation of diffusion chambers

Diffusion chambers were composed of 2 14-mm lucite rings, covered on 1 side with 3.0-μm SSWP membranes or 5.0-μm hydrophilic Durapore membranes, cemented to each other (Millipore, Bedford, Massachusetts), and then heated to 75 C for 4 hr. Larvae, in lots of 20, were placed into a syringe equipped with a 23-gauge needle, and inoculated into a diffusion chamber through a hole in one of the rings; the hole was subsequently sealed with nylon thread.

Laboratory conditioned, random source beagles, male and female BALB/c mice and male C57BL/6 and DBA/2 mice (Charles River, Wilmington, Massachussetts or Jackson Laboratories, Bar Harbor, Maine) received implants of diffusion chambers. Dogs were anesthetized and a subcutaneous pocket was formed in the ventral skin of the neck into which 7–15 diffusion chambers were placed. Diffusion chambers implanted into 5 dogs were removed from 1 dog 1 wk postim-

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* Present address: Department of Microbiology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107.

† Pitman-Moore, Inc., Washington Crossing, New Jersey.

plantation and from 2 dogs 2 wk postimplantation and 2 dogs 3 wk postimplantation. Mice were anesthetized prior to implantation of a single chamber in a subcutaneous pocket formed lateral to the posterior spine. After 1–3 wk, diffusion chambers were removed from dogs or mice and all live larvae were enumerated and placed into glacial acetic acid. In only rare instances were dead larvae observed in diffusion chambers recovered from control or immunized animals. Dead larvae were therefore not evaluated in these studies. The larvae were then immersed in 70% ethanol containing 5% glycerine that was allowed to evaporate, leaving the larvae cleared in glycerine. The worms were measured by tracing their projected images with an opisometer. Infiltrates within diffusion chambers were histologically prepared using standard procedures. These tissues were stained with hematoxylin–eosin or with Mallory's phosphotungstic acid hematoxylin.

Immunization methods

Male BALB/c mice were used in all of the immunization experiments. Challenge infections consisted of larvae in diffusion chambers implanted in mice for 3 wk. Mice were immunized by subcutaneous injection of live L3 using the following regimens:

A) Day 1: 50 L3, Day 7: 25 L3, Day 14: 15 L3, Day 21: 10 L3, Day 28: 10 L3, Day 35: 10 L3, Day 42: challenge (total L3 = 120);
B) Days 1, 7, 14, 21, 28, 35: 30 L3, Day 42: challenge (total L3 = 180);
C) Day 1: 100 L3, Day 21: 100 L3, Day 42: challenge (total L3 = 200);
D) Day 1: 300 L3, Day 21: 150 L3, Day 42: challenge (total L3 = 450); and
E) Day 1: 75 L3, Day 14: 75 L3, Day 21: 75 L3, Day 28: challenge (total L3 = 225).

Larvae were also injected intravenously, following Regimen B described above. Larvae killed by freezing and larvae that were attenuated by treatment with 20 krad gamma-radiation from a cesium-137 source were also used to immunize mice following Regimen E described above.

Immunization was also attempted with dead larvae plus adjuvants. Mice were immunized with 150 L3 in Freund's complete adjuvant (Day 1) followed by 150 L3 administered intravenously (Day 15). Challenge occurred on Day 21 and diffusion chambers were removed on Day 32. Another approach to immunization used freeze-thawed L3 and L4 injected intradermally. L4 were obtained 8 days postinoculation from an *in vitro* culture system consisting of NCTC and IMDM media mixed 1:1 supplemented with 20% fetal calf serum. One hundred L3 or L4 were injected into mice in 0.1 ml PBS with or without 5×10^6 colony-forming units of *Mycobacterium bovis* (BCG) (ITR Biomedical Research, Chicago, Illinois). BCG without L3 or L4 was also injected into mice as a control. Four weeks after intradermal immunization, mice were challenged with either L3 or L4 and diffusion chambers were removed 1 wk later.

Antibody assays

Sera for use in antibody assays were collected from control and immunized mice when the diffusion chamber challenge infections were recovered. Antibodies directed against the surface antigens of L3 were studied using L3 fixed in 4% formalin. After fixation larvae were washed in PBS and incubated in mouse serum for 30 min at 37 C. Larvae were washed in PBS and incubated in an FITC-conjugated, IgG fraction of goat anti-mouse μ chain or γ chain (Cooper Biomed, West Chester, Pennsylvania). After washing in PBS, larvae were placed in PBS with 25% glycerine and 0.1% p-phenylenediamine. Intensity of fluorescence was measured on 3 representative larvae/serum sample using a Nikon P-1 photometer (Nikon, Tokyo, Japan). A mean was obtained of the 3 readings and the results were expressed as relative fluorescence units (RFU).

Soluble L3 and L4 antigens were prepared by placing larvae in cold PBS with 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 25 μg/ml leupeptin, and then sonicating the larvae for 2 min. Larvae were extracted for approximately 24 hr at 4 C after which the preparations were centrifuged, supernatant collected, and protein content determined. Antibody levels to soluble L3 and L4 antigen were assessed with an indirect enzyme-linked immunosorbent assay (ELISA), using horseradish peroxidase-conjugated goat anti-mouse γ, μ, and α chain (Cooper Biomed, West Chester, Pennsylvania). Reactions were evaluated with a semi-automated spectrophotometric reader (Bio-Tek, Burlington, Vermont).

Monoclonal antibody preparation and analysis

Mice received intraperitoneal injections of freeze-thawed L3 in Freund's complete adjuvant on Days 0 and 14 and an intravenous injection of soluble L3 antigen on Day 25. Spleen cells recovered 4 days later were fused with P3/X63/Ag8 myeloma cells and hybridomas prepared according to the method of Kohler and Milstein (1975). Supernatant from hybridomas were screened for reactivity with *D. immitis* L3 surface antigens in an indirect fluorescent antibody assay as described above. Isotype identification of the monoclonal antibody was performed using a sub-isotype kit (Hyclone, Logan, Utah).

Specificity of the monoclonal antibody was assessed using the following antigen preparations: (1) cryostat sections of *D. immitis* L3, (2) cryostat sections of adult *D. immitis,* (3) intact fixed *D. immitis* L4 obtained from *in vitro* culture, (4) intact fixed *D. immitis* microfilariae, (5) intact fixed *Dipetalonema viteae* L3 obtained from ticks, *Ornithodorus tartakovski,* (6) intact fixed *D. viteae* microfilariae from jirds, (7) intact fixed *Brugia malayi* L3 from *A. aegypti,* (8) intact fixed *B. malayi* microfilariae from jirds, (9) intact fixed *Onchocerca lienalis* L3 from *A. aegypti* (Lok et al., 1980), and (10) intact fixed *O. lienalis* microfilariae from bovine skin. All of these filarial worm preparations were tested for reactivity with the monoclonal antibody by an indirect fluorescent antibody assay.

In vivo administration of monoclonal antibody

For the production of ascites, mice were injected with 0.5 ml of 2,6,10,14-tetramethyl-pentadecane (pristane) intraperitoneally 1 wk prior to intraperitoneal administration of 5×10^6 hybridoma cells. Ascites containing monoclonal antibody was injected (0.33 ml/mouse) either intravenously or subcutaneously into mice

TABLE I. *Survival and growth of larval* Dirofilaria immitis *in diffusion chambers implanted into dogs.*

Weeks implanted	No. chambers	% Recovery	No. larvae measured	Larval length (μm)*
1	7	73 ± 33†	50	1,151 ± 78
2	12	57 ± 33	26	1,265 ± 120
3	25	44 ± 22	49	1,330 ± 130

* Length of L3 recovered from mosquito = 809 ± 68.
† Mean ± standard deviation.

with a concomitant implantation of a diffusion chamber containing L3. Ascites containing IgM monoclonal antibody against the surface and excretory–secretory antigens of *Toxocara canis* infective larvae was injected into control mice concomitant with larval challenge. Diffusion chambers were recovered 1 or 2 wk later and their contents evaluated for live larvae as described above.

Statistical analysis

Analysis of data obtained from active immunization studies using various immunization regimens was performed with Student's *t*-test. Data obtained on surface- and soluble-antigen-associated antibody levels, and challenge worm recoveries for mice immunized with live, irradiated, and killed larvae were analyzed using analysis of variance followed by Scheffe's multiple comparisons and multiple regression. Monoclonal antibody passive immunization experiments were analyzed by 3-factor analysis of variance. Statistical significance was determined at $P < 0.05$.

RESULTS

Larval *D. immitis* implanted in diffusion chambers

Larval *D. immitis* survived and developed in diffusion chambers implanted in dogs. During the 3-wk period of implantation, larval lengths increased, whereas the proportion of larvae surviving decreased (Table I). A thick fibrous wall formed around diffusion chambers implanted in dogs, and a dense cellular infiltrate filled the chambers.

Dirofilaria immitis L3 developed in diffusion chambers implanted in the 3 inbred strains of mice selected for study. Larvae grew and survived for 2 wk, at equivalent rates, regardless of the sex or strain of mouse tested. An increase in larval length with a concomitant decrease in larval survival was noted during the 3-wk implantation in BALB/c mice (Table II). Diffusion chambers implanted in mice induced little or no tissue encapsulation. A fibrin matrix, as evidenced by Mallory's phosphotungstic acid hematoxylin staining, formed inside chambers in which inflammatory cells were dispersed.

TABLE II. *Survival and growth of larval* Dirofilaria immitis *in diffusion chambers implanted into mice.*

Strain	Sex	No. mice	No. weeks implanted	% Recovery	No. larvae measured	Larval length (μm)*
BALB/c	M	9	1	89 ± 11†	28	1,218 ± 117†
	M	12	2	85 ± 13	27	1,360 ± 102
	M	28	3	80 ± 16	29	1,474 ± 157
BALB/c	F	7	2	81 ± 14	47	1,351 ± 114
C57BL/6	M	8	2	81 ± 10	77	1,326 ± 126
DBA/2	M	8	2	82 ± 8	39	1,289 ± 120

* Length of L3 recovered from mosquito = 809 ± 68
† Mean ± standard deviation.

Active immunization of mice

BALB/c mice, inoculated with normal live L3 using a variety of regimens, demonstrated a significant reduction in survival of challenge larvae. In only rare instances were dead larvae observed, in diffusion chambers recovered from control or immunized mice, and no consistent patterns of cellular adherence were observed. No apparent differences in ability to generate protective immunity were observed among the various regimens employed (Table III). Larvae recovered from normal and immunized mice were measured and no difference in growth rates was observed. Normal, irradiated, and killed L3 were repeatedly injected into mice and the mice were subsequently challenged with L3 in diffusion chambers. No diminution in larval survival was seen in mice immunized with dead larvae; apparently, antigens associated with live L3 and/or L4 are needed to generate protective immunity) (Table IV). To further test the apparent inability of dead larvae to stimulate protective immune responses, killed L3 alone or with either BCG or Freund's complete adjuvant, and killed L4 alone or with BCG were inoculated into mice. The mice were then challenged with chambers containing either L3 or L4. No reduction in chal-

TABLE III. *Immunization and challenge of BALB/c mice with L3 of* Dirofilaria immitis.

Regimen	No. larval inoculations	Total no. larvae	Immunization route	No. mice	% Larval recovery
—	0	0	—	12	76 ± 18*
A	6	120	Subcutaneous	6	53 ± 31
B	6	180	Subcutaneous	5	57 ± 12
B	6	180	Intravenous	6	53 ± 12
C	2	200	Subcutaneous	5	46 ± 33
D	2	450	Subcutaneous	5	54 ± 12

* Mean ± standard deviation

TABLE IV. *Immunization of BALB/c mice using regimen E with normal, irradiated, and killed* Dirofilaria immitis *L3; effects on challenge larval recovery and antibody production determined 3 wk after challenge.*

Condition of immunizing larvae	% Recovery	Antibody levels to L3 surface* IgG	Antibody levels to L3 surface* IgM	Antibody levels to Soluble† L3	Antibody levels to Soluble† L4
—	78 ± 14‡	2.7 ± 0.6	6.4 + 5.9	24.2 ± 22.9	35.9 ± 25.9
Normal	58 ± 9	2.7 ± 0.2	8.4 ± 5.3	86.4 ± 40.5	150.2 ± 58.6
Irradiated	43 ± 13	2.0 ± 0.1	6.0 ± 3.4	111.5 ± 64.5	126.0 ± 43.6
Killed	86 ± 19	2.7 ± 0.4	8.2 ± 3.3	76.8 ± 34.9	129.6 ± 25.0

* Relative fluorescent units.
† Absorbance values of ELISA at 405 nm.
‡ Mean ± standard deviation.

lenge larval survival was found in any of the mice immunized with dead larvae.

Antibody levels and protective immunity

Humoral responses to the surface of L3 and to soluble extracts of L3 and L4 were assessed in mice receiving normal, irradiated, and killed larval immunizations. No statistically significant differences were noted in the IgG and IgM L3-surface-specific antibody levels of normal and immunized mice. There was, however, an inverse relationship between antibody levels to soluble L3 ($r^2 = 33.9$) and L4 ($r^2 = 16.1$) antigens and larval recoveries (Table IV). This was observed when the data were analyzed not separating the various immunization regimens in the statistical analysis. However, when the data from individual immunization regimens were analyzed as groups, only the association between antibody titer to soluble L3 antigen and larval recovery remained statistically significant ($r^2 = 63.9$) (Fig. 1).

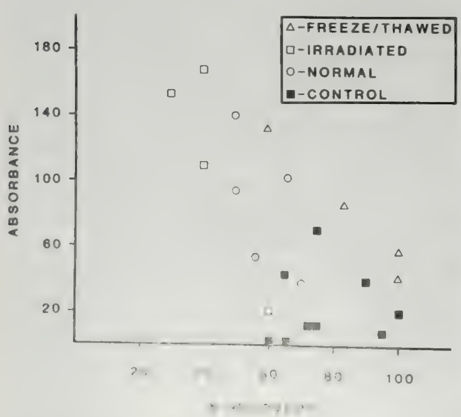

FIGURE 1. Relationship between antibody titer to soluble L3 antigen and challenge larval recovery from BALB/c mice immunized against *Dirofilaria immitis* with normal, irradiated, or killed L3.

Monoclonal antibody to L3 surface

A monoclonal antibody (229) of IgM isotype was generated that was reactive with the surface of *D. immitis* L3. It was not reactive with the somatic tissues of L3 nor with the surfaces of L4, adults, or microfilariae of *D. immitis* (Figs. 2, 3). Third-stage larvae and microfilariae of *O. lienalis, B. malayi,* and *D. viteae* were tested for reactivity with the monoclonal antibody, and of these worms, only *O. lienalis* L3 bound the antibody to its surface.

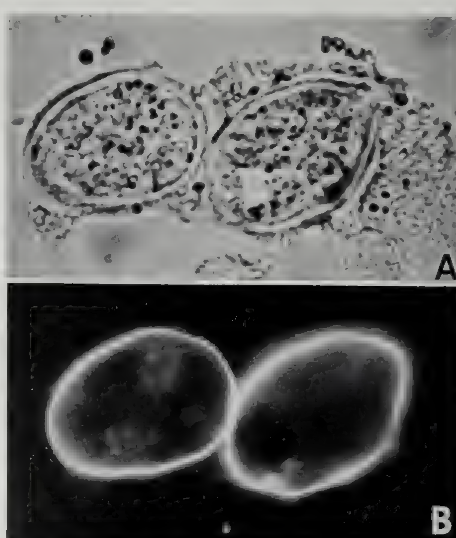

FIGURE 2. **A.** Cryostat section of *Dirofilaria immitis* L3 incubated with monoclonal antibody 229, followed by FITC-conjugated anti-antibody viewed with light microscopy. **B.** As above, viewed with fluorescent microscopy.

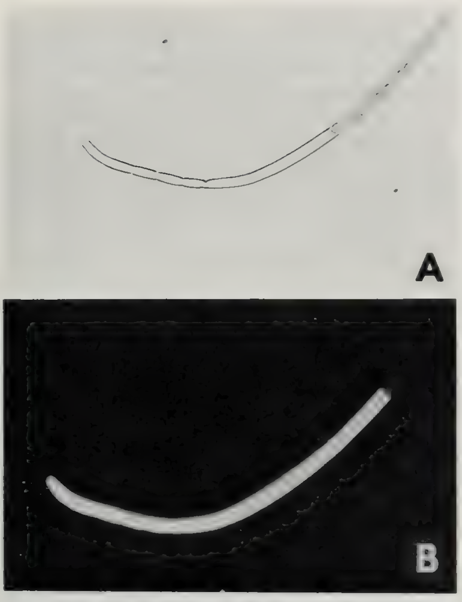

FIGURE 3. **A.** L4 of *Dirofilaria immitis* escaping from L3 cuticle incubated with monoclonal antibody 229 followed by FITC-conjugated anti-antibody viewed with light microscopy. **B.** As above, viewed with fluorescent microscopy.

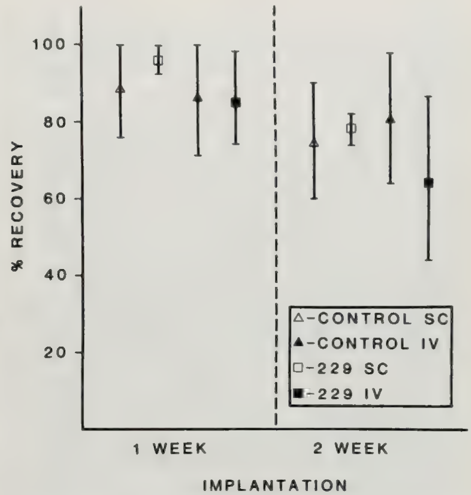

FIGURE 4. Survival of *Dirofilaria immitis* larvae in mice for 1 or 2 wk after intravenous or subcutaneous inoculation of monoclonal antibody 229 or a monoclonal antibody reactive with larval *Toxocara canis*.

Passive immunization of mice

Ascites containing monoclonal antibody 229 or an IgM monoclonal antibody directed against larval *T. canis* surface and excretory–secretory antigens was injected subcutaneously or intravenously into mice with a concomitant implantation of a diffusion chamber containing L3. A small reduction in larval recovery was observed in mice that received intravenous antibody when implanted diffusion chambers were retained for 2 wk. This reduction was, however, not statistically significant (Fig. 4). Larvae, recovered from mice receiving specific or nonspecific monoclonal antibody, were measured with no difference in growth observed.

DISCUSSION

The present study has demonstrated that larval *D. immitis* will survive and develop for at least 3 wk within diffusion chambers implanted in mice or dogs. Discrepant findings have been reported on whether mice are susceptible to infection with larval forms of *D. immitis.* Ohgo (1980) inoculated L3 subcutaneously into ddy and C57/BL mice and recovered live larvae for up to 4 wk. Delves and Howells (1985) injected L3 intraperitoneally into several strains of suckling mice, including C57BL/10 and BALB/C, and found all larvae were eliminated within 24 hr. However, when L3 were implanted in mice in diffusion chambers, larvae were recovered live. It thus remains unclear whether mice will maintain larval *D. immitis* outside of diffusion chambers; however, within diffusion chambers *D. immitis* larvae will develop and grow in mice.

Delves and Howells (1985) compared the rates of survival and molting of *D. immitis* L3 after 74 hr in diffusion chambers implanted in mice and implanted in a naturally susceptible host, the ferret, and no differences were observed. Furthermore, it was reported that worms recovered from mice after 8 days had growth rates comparable to that found in larvae recovered from infected dogs. In the present study, larvae were implanted in mice and dogs for up to 21 days. At 3 wk postimplantation, no differences were noted between the lengths of larvae recovered from diffusion chambers implanted in mice or dogs. The lengths of worms recovered from diffusion chambers, however, were smaller than that reported for worms recovered from dogs infected for 3 wk (Lichtenfels et al., 1985).

Mice immunized with live larvae in a variety of dosages and schedules were capable of eliminating a significant proportion of challenge in-

fections. Larvae attenuated by irradiation (Wong et al., 1974) were also capable of eliciting a protective immune response. Numerous filarial worm infections have been shown to be significantly reduced by protective immunity induced in the host by live or irradiation-attenuated L3 (Wong et al., 1974; Gass et al., 1979; Tanner and Weiss, 1981a; Yates and Higashi, 1985; Abraham et al., 1986). Ohgo (1980) immunized C57BL mice with normal *D. immitis* L3 and demonstrated an elimination of 98% of challenge larvae as compared to controls. The discrepancy between the levels of parasite elimination seen in the present study and that reported by Ohgo (1980) may be a result of different strains of mice utilized, strains of parasite used, immunization protocols, or the method of challenge larval recovery. Both studies do clearly demonstrate that mice are capable of developing acquired protective immune responses to larval *D. immitis.*

Larvae recovered from chambers implanted in immunized mice did not show any signs of growth retardation in comparison to worms from control animals. Retardation of filarial–larval growth in immunized hosts has been reported for *Litomosoides carinii* (Scott and MacDonald, 1958; Weiner et al., 1984) and *Dipetalonema viteae* (Gass et al., 1979; Tanner and Weiss, 1981a; Abraham et al., 1986). It is unknown whether *D. immitis* larvae do not induce, or are not susceptible to, immune-mediated growth retardation, or whether mice are incapable of generating this response.

Several studies have demonstrated that protective immunity to helminth infections can be elicited with dead worms. Dead *D. viteae* L3 are capable of inducing destruction of challenge larvae at a level similar to that induced by live larvae (Tanner and Weiss, 1981a). Dead *L. carinii* L3 in Freund's complete adjuvant induced high levels of resistance to homologous infection (Mehta et al., 1981b). Freeze-killed cercariae of *S. mansoni,* administered intradermally with BCG have also been shown to induce resistance (James, 1985). In the present study immunizing mice with killed L3 alone or in Freund's complete adjuvant, or with either killed L3 or L4 alone or with BCG did not produce evidence of immunity against subsequent L3 or L4 challenge. It appears that antigens capable of eliciting a protective immune response are only associated with live larvae; perhaps these antigens are present in the excretory–secretor [illegible] the live worms.

Serum alone or with cells has been shown to be effective at killing filarial larvae *in vitro* (Higashi and Chowdhury, 1970; Subrahmanyam et al., 1978; Mehta et al., 1981a; Tanner and Weiss, 1981b; Sim et al., 1982; Chandrashekar et al., 1985; Haque et al., 1985). It was of interest, therefore, to determine whether there was a relationship between the levels of immune resistance and specific antibody found in immunized mice. No correlation was seen between immunity and L3-surface-specific antibody levels; a correlation did exist between antibody titer to soluble L3 and L4 antigens and worm recovery.

All sera in the present study were collected at the time challenge diffusion chambers were recovered. It is possible that the antibody responses to L3 and L4 antigens observed in these experiments were a response to released antigens from killed larvae found within the diffusion chambers and therefore are a poor reflection of what precipitated the elimination. Larval *T. colubriformis,* for example, have been shown to be capable of eliciting systemic immune responses from within diffusion chambers (Rothwell and Love, 1974). Sequential quantitation of serum antibody levels to a variety of specific antigen types is therefore necessary to help define, by correlation, the role of antibody in protective immunity.

Wong et al. (1974) have described a relationship between the presence of protective immunity to *D. immitis* in dogs and the antibody response to the surface of L3. Furthermore, monoclonal antibodies reactive with the surface of several helminth parasites have been shown to be protective in passive transfer studies (Canlas et al., 1984; Ortega-Pierres et al., 1984; Aggarwal et al., 1985; Hazdai et al., 1985). To further test the role surface-reactive antibodies play in protective immunity, a monoclonal antibody (229), directed against L3 surface, was developed for passive transfer studies. These antibodies were found to be reactive with surface of *D. immitis* and *O. lienalis* L3, but not the surfaces or somatic tissues of various other filarial species and stages.

Passive transfer of monoclonal antibody 229 did not, however, produce statistically significant protection. Under one set of conditions, i.e., intravenous administration of antibody and allowing challenge diffusion chambers to remain implanted for 2 wk, some reduction of parasite survival was noted. It is possible that the conditions under which the antibody was administered were not optimal for its effective elimina-

tion of the parasites. Alternatively, it may be that the isotype or binding site of the monoclonal antibody was inappropriate or that surface-reactive antibodies are not important in protective immune responses to larval *D. immitis* in mice. The absence of correlation between anti-L3-surface antibodies and protection described in the present study would support the latter possibility.

In conclusion, the active immunization studies have shown that live larvae are needed to generate protective immunity, and that, whereas there is no relationship between immune protection and antibodies to larval surfaces there is a relationship between protection and antibodies to soluble larval antigens. Furthermore, the surface-directed monoclonal antibody tested in this study proved ineffective at protecting mice from challenge infections.

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TRICHINELLA SPIRALIS INFECTIONS OF INBRED MICE: IMMUNOLOGICALLY SPECIFIC RESPONSES INDUCED BY DIFFERENT *TRICHINELLA* ISOLATES

Donald L. Wassom, Debra A. Dougherty*, and Terry A. Dick†
Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706

ABSTRACT: The immune response of inbred mice was studied following infection with *Trichinella spiralis* var. *pseudospiralis* (TP) or with isolates of *T. spiralis* derived from a pig or from an arctic fox. Animals given a primary infection with 1 isolate of *Trichinella* and challenged 21 days later with the same or different isolates responded more quickly by expelling worms from the homologous challenge. In addition, although mesenteric lymph node cells from mice infected with each isolate of *Trichinella* would proliferate *in vitro* when cultured with antigen derived from each of the others, the strongest proliferation response always occurred when cells were cultured in the presence of antigen prepared from the specific isolate used to infect the mouse from which the cells were derived. In addition, it was possible to prepare monoclonal antibodies that recognized an antigen expressed by TP which was not shared by *T. spiralis* isolates and vice versa. Collectively, these data support the conclusion that the differences observed in the kinetics of immune responsiveness to different *Trichinella* isolates are referable, at least in part, to differences among the isolates in the expression of functionally relevant antigens.

Because of conflicting reports in the literature regarding the outcome of experimental *Trichinella* infections in inbred strains of mice, we were curious to determine if different *Trichinella* isolates might interact differently with the immune system of a common inbred strain of mouse. A recent study by Palmas et al. (1985) addressed this question and they concluded that the host response to *T. spiralis* and *T. pseudospiralis* was very similar; immunity to *T. pseudospiralis* and *T. spiralis* could be stimulated in NIH mice by prior infection with either parasite, by injection of *T. spiralis* larval antigen, and by adoptive transfer of immune mesenteric lymph node cells taken from mice infected with either parasite. In addition, it was reported that the strength and kinetics of the blast cell responses of B10.G and NIH mice infected with either parasite correlated with the kinetics of the response that expelled worms from the gut. In the present report we demonstrate that although different *Trichinella* isolates may share relevant antigens, they also possess antigens which appear to be unique, and that these unique antigens are functionally relevant to the outcome of experimental infections.

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* Present address: Department of Microbiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853.

† Present address: Department of Zoology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.

MATERIALS AND METHODS

Mice

All mice used in these experiments were 5–8-wk-old males reared in the immunogenetics mouse colony at Cornell University. Mice were housed 5 per cage in 29.5 × 18.5 × 13-cm polycarbonate cages and fed Charles River RMH 1000 formula *ad libitum*. All mice were kept on a photoperiod of 12 hr daylight, 12 hr darkness.

Trichinella isolates

Trichinella spiralis var. *pseudospiralis* (TP) was obtained from Dr. G. Faubert (MacDonald College, McGill University). *Trichinella spiralis* isolates from an infected pig (P_1 = pig; 43°00′W; 1952) and from an arctic fox (AF_1 = arctic fox_1; 69°15′N, 105°00′W; 1980) were also tested.

Infections

The infective muscle larvae from each of the *Trichinella* isolates were obtained from carcasses of freshly killed C3HeB/FeJ male mice. Mice were killed by cervical dislocation, skinned and eviscerated, and minced individually in a Waring blendor containing 100 ml of 1% HCl and 1% pepsin (P7000, Sigma Chemical Co., St. Louis, Missouri). Each minced carcass, along with an additional 100 ml of acid–pepsin solution, was transferred to a 250-ml polycarbonate flask and incubated for 2 hr at 37 C in an Orbit Environmental Shaker (Labline Inc.) at 200 rpm. After digestion, the worms were isolated from remaining debris by passing the material over a 100-mesh sieve (5-in. diameter) and collecting the larvae on a 250-mesh sieve stacked below it. Larvae were washed from the lower sieve into 50-ml conical centrifuge tubes using 0.85% NaCl and washed 5 times by resuspending the larvae in saline and allowing them to settle out of solution at 1 *g*. All experimental mice were infected per os with 150–200 muscle larvae using a 0.5-ml syringe equipped with a

blunt, curved, 18-gauge dosing needle. Mice used as donors of infective larvae had been infected 30–45 days previously. The use of a single, inbred strain of mouse as a donor of larvae for all infections served to control for the possibility that the genetic composition of the mice used as donors of larvae may influence subsequent establishment of the parasite in the new host.

Adult counts

Mice were killed by cervical dislocation and the entire small intestine was immediately removed to a 100-mm plastic petri dish containing 25 ml of warm phosphate-buffered saline, pH 7.2 (PBS). The small intestine was slit longitudinally along its entire length and incubated at 37 C for at least 4 hr. The intestine was then discarded and the worms, which migrate out of the tissues and into the saline, were counted using a dissecting microscope.

Antigens

Infective muscle larvae from each of the *Trichinella* isolates were freed of all muscle tissue by acid–pepsin digestion and the worms were washed 8 times in 0.85% NaCl by allowing the well-mixed larvae to settle out of suspension at 1 *g*. The antigen extracts were prepared by homogenizing washed muscle larvae in a Tenbrock glass tissue homogenizer (Kontes Glass). The viscous extract was centrifuged at moderate speed to remove large debris and the supernatant was saved and centrifuged again (40,000 *g*). The supernatant from the second centrifugation was dialyzed against 3 changes of phosphate-buffered saline (pH 7.2) using a 5,000 MW cutoff membrane (Spectrapore, Spectrum Medical Instruments, Inc.) and then centrifuged again at 40,000 *g*. The supernatant from the final centrifugation was assayed for protein concentration using the Bio-Rad protein assay kit and aliquots were stored at −80 C until used.

Lymphocyte proliferation assay

Mice were killed by cervical dislocation, swabbed with 70% ethanol, the peritoneal cavity opened, and the mesenteric lymph nodes removed aseptically to 17 × 100-mm tubes (Falcon no. 2201) containing 5.0 ml Hanks' balanced salt solution (HBSS) supplemented with 1 M HEPES (GIBCO no. 380-5630), penicillin–streptomycin solution (GIBCO no. 600-5140), 30 mg/L DNase (Sigma no. D0876), and 2% horse serum (GIBCO). Single cell suspensions from the mesenteric lymph nodes were prepared at room temperature by gently crushing the lymph node using a teflon-tipped tissue grinder adapted to fit snugly into the culture tube. After waiting several minutes for large debris to settle, the cells were removed via sterile pasteur pipets to 15-ml conical centrifuge tubes (Corning no. 25311) and washed 3 times in supplemented HBSS by centrifugation at 400 *g*. After the final wash, cells were suspended in 2.2 ml culture medium containing RPMI-1640 (GIBCO no. 330-2511), 1 M HEPES, penicillin–streptomycin solution, 200 mM glutamine (Flow no. 16-801-46), 3 × 10^{-5} M 2-mercaptoethanol (Sigma no. M-6250), 5% horse serum (GIBCO) (pH 7.2). Cells were counted using a Coulter counter (Coulter Electronics, Model Z_f) and viability determined by trypan blue exclusion. Each cell suspension was dilu

ml and 4 × 10^5 cells in 0.1 n

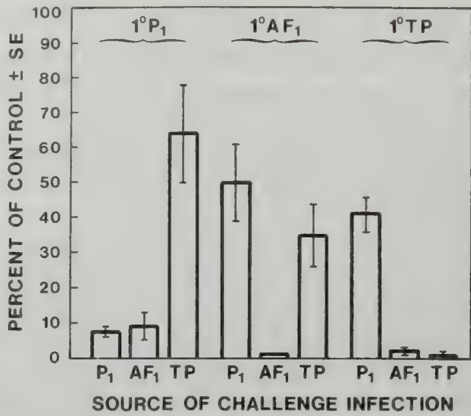

FIGURE 1. Immunity to challenge infections with different *Trichinella* isolates. B10.M mice were infected per os with 150–200 L_1 larvae of either the P_1, AF_1, or TP isolates of *Trichinella* as shown by brackets at the top of the figure. Twenty-one days later groups of 6 mice each were challenged with either the P_1, the AF_1, or the TP isolates. Five control mice received a primary infection with each isolate at the time that experimental mice were challenged. Six days later worms in the small intestine of challenge and control mice were counted. Counts for challenged mice are expressed as a percentage of the mean worm count obtained for the primary infection controls.

12 wells of a 96-well, sterile, microtiter plate (Costar no. 3596). Serum-free cell culture medium (0.1 ml) was added to the first 3 wells, and *Trichinella* antigen (see above) at 100, 200, or 400 μg/ml in 0.1 ml of serum-free cell culture medium was added to each of 3 wells, respectively.

After 4 days culture, [^{3}H] thymidine (1.6 μCi in 0.01 ml HBSS) was added to each well, and 24 hr later the cells were harvested onto filter paper discs. The filter paper was air-dried and the individual discs punched into 13 × 57-mm vials (Wheaton 225402) containing 3 ml scintilline (Fisher SO-X-2). Disintegrations per minute were counted using a Beckman LS 7000 scintillation counter: counts were obtained from the 3 samples at each antigen concentration, and the increase in [^{3}H] thymidine uptake (Δcpm) was calculated by subtracting the average background count for the unstimulated controls from the value for each sample.

RESULTS

Three groups of 18 B10.M mice were infected with 150–200 L_1 larvae of either TP, P_1, or AF_1. Twenty-one days later, each group of 18 mice was divided into equal groups of 6 mice each and challenged with either the P_1, the AF_1, or the TP isolates of *Trichinella*. Six days following the challenge infection, the mice were killed and worms in the small intestine were counted. Worm counts shown in Figure 1 are expressed as a per-

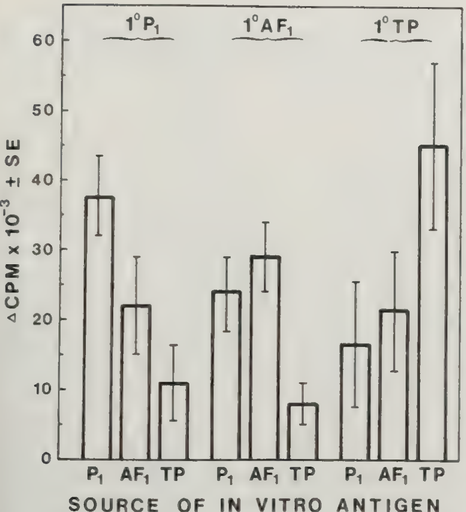

FIGURE 2. Proliferative response of mesenteric lymph node cells harvested from B10.M mice infected with different *Trichinella* isolates. B10.M mice were infected per os with 150–200 L_1 larvae of either the P_1, AF_1, or TP isolates of *Trichinella* as shown by brackets at the top of the figure. Six days later mice were killed and single cell suspensions were prepared from the mesenteric lymph nodes. Cells were cultured *in vitro* in the presence of antigens prepared separately from the P_1, AF_1, and TP isolates of *Trichinella*. Values for Δcpm were obtained by subtracting the counts obtained from cells cultured in medium alone from the cpm obtained from cells cultured with the different *Trichinella* antigens. Cells from uninfected mice did not proliferate in response to these antigen preparations.

centage of the mean worm count obtained from groups of 5 primary infection control mice infected at the time of challenge. The numbers of worms establishing in the small intestine did not differ significantly among the *Trichinella* isolates.

Mice, primed by infection with any of the 3 isolates, were significantly protected against challenge with each of the others. However, there were marked differences in the degree of protection induced. For example, mice primed with P_1 resisted a challenge with both P_1 and AF_1 but were quite susceptible to reinfection with TP. Mice primed against AF_1 resisted reinfection with the homologous isolate much better than challenge with P_1 or TP. To our surprise, mice infected initially with TP were strongly resistant to challenge with TP and AF_1 but comparatively susceptible to challenge with P_1.

These data support the conclusion that different *Trichinella* isolates share antigens of functional importance, but suggest that qualitative or quantitative differences in these or other antigens render each isolate immunologically distinct. To explore this question with more precision, we studied the *in vitro* proliferation response of mesenteric lymph node cells harvested from infected mice and cultured in the presence of antigens prepared separately from each of the 3 *Trichinella* isolates. In the first experiment, 24 B10.M mice were divided into 3 groups of 8 mice each and infected with either the P_1, AF_1, or TP isolates of *Trichinella*. Six days later the mesenteric lymph node cells were removed from these mice and 4 pools of cells from 2 mice each were cultured in the presence of antigen prepared from the homologous as well as each of the heterologous isolates. Results of this experiment are shown in Figure 2. In each case, the strongest proliferation response occurred when cells were cultured in the presence of homologous antigen. For example, cells harvested from TP-infected mice proliferated poorly in the presence of P_1 or AF_1 antigen when compared to the vigorous response induced when cultured with TP antigen.

In a second series of experiments we compared the proliferation of mesenteric lymph node cells harvested from B10.BR mice on day 9 postinfection. It is known that B10.BR mice are susceptible to pig isolates of *T. spiralis* when compared to the B10.M mice studied in the earlier experiments (Wassom et al., 1984). Results are shown in Figure 3. As was seen in the previous experiment, cells from mice infected with 1 isolate of *Trichinella* proliferated most strongly when cultured with the homologous antigen.

Results of our *in vitro* studies confirmed that each of the *Trichinella* isolates tested was antigenically distinct. In the case of P_1 and TP, further studies confirmed that each possessed at least 1 antigen not shared by the other. Balb/c mice were injected with either P_1 or TP antigen preparations, and spleen cells from these mice were fused with P3×63 Ag 8.653 myeloma cells in the presence of polyethylene glycol to establish hybridoma cell lines. Hybridoma cells making antibody reactive with the injected antigens were cloned from these cell populations and tested in an ELISA against antigens prepared from either P_1 or TP. We identified several cell lines making monoclonal antibody reactive with P_1 but not TP; 1 monoclonal antibody recognized an antigen expressed by TP but not P_1, at least not in quantities detectable in our ELISA assay.

DISCUSSION

At first glance our results appear to contradict the published results of Palmas et al. (1985) who compared an isolate of *T. spiralis* with an isolate of *T. pseudospiralis* and concluded that these worms showed extensive cross reactivity. We do not dispute this conclusion. Our results likewise suggest that *T. spiralis* and *T. spiralis* var. *pseudospiralis* share functionally relevant antigens. However, we believe that the experiments of Palmas et al. (1985) were not optimally designed to detect antigenic differences that might occur between parasites. For example, although they studied *T. spiralis* and *T. pseudospiralis* infections in slow responder (B10.G) and fast responder (NIH) mice, they noted marked differences in the kinetics of the expulsion response only in the slow responder strain. Yet all subsequent *in vivo* experiments designed to study reciprocal immunity to challenge infections, injections of parasite antigens, or adoptive transfer of immunity were conducted exclusively in fast responder NIH mice—the strain shown to exhibit only minimal differences in responsiveness to the 2 parasite isolates.

Studies by Palmas et al. (1985) of the *in vitro* blast cell response to *Trichinella* antigen employed only antigen prepared from *T. spiralis* L_1 larvae; antigen prepared from *T. pseudospiralis* was never tested. In addition, the stimulation indices reported for the blast cell response to *Trichinella* antigen were extremely low. This must reflect either poor stimulation of the cultured cells or very high background counts in control cultures. We have chosen to report our cell proliferation results in terms of Δcpm rather than as a stimulation index. However, if we were to use a stimulation index calculated in the manner described by Palmas et al. (1985), our indices would range 10–100-fold higher than theirs. It is likely that cell cultures showing low backgrounds and strong specific proliferation responses will be more sensitive indicators of differences between the antigens used to stimulate them.

Our data support the conclusion that the differing outcomes of experimental infections with P_1, AF_1, and TP (Dick et al., 1986) may be due, at least in part, to the differential expression of functionally relevant antigens. Our results extend observations made in comparative studies of *T. spiralis*- and *T. pseudospiralis*-infected mice to studies of mice infected with *T. spiralis* isolates derived from different host species. It should be noted however that we observed no significant differences in the outcome of experimental infections initiated with *T. spiralis* isolates originating from different pigs (unpubl. data); it is therefore unlikely that disparate results emanating from different laboratories studying infections with pig isolates can be attributed to significant differences, antigenic or otherwise, in the way that these parasites interact with a common inbred strain of host.

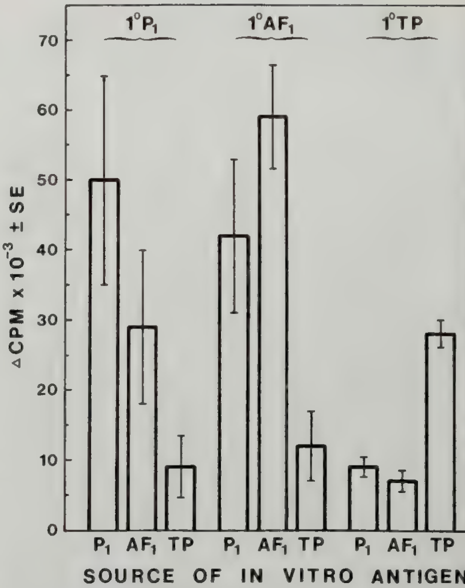

FIGURE 3. Proliferative response of mesenteric lymph node cells harvested from B10.BR mice infected with different *Trichinella* isolates. Cells were harvested from infected mice on day 9 postinfection. Otherwise, experimental protocol is as outlined in Figure 2.

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CULTIVATION OF *CRYPTOSPORIDIUM BAILEYI*: STUDIES WITH CELL CULTURES, AVIAN EMBRYOS, AND PATHOGENICITY OF CHICKEN EMBRYO-PASSAGED OOCYSTS

David S. Lindsay*, Christine A. Sundermann†, and Byron L. Blagburn
Department of Pathology and Parasitology, College of Veterinary Medicine, Auburn University, Auburn, Alabama 36849

ABSTRACT: Sporozoites of *Cryptosporidium baileyi* did not undergo development in primary cell cultures from either avian or mammalian hosts, or in mammalian cell lines. Oocysts of *C. baileyi* produced infections resulting in complete development to sporulated oocysts in chicken embryos and embryos of 8 other avian species examined. Inoculation of 4×10^5 oocysts was not pathogenic for avian embryos as evidenced by the lack of gross lesions or death. Oocysts obtained after *C. baileyi* had been passaged 10 times (first experiment) or 20 times (second experiment) in chicken embryos still caused clinical respiratory disease and gross airsacculitis when inoculated intratracheally into 2-day-old broiler chickens. Oocysts that had been passaged 10 times in chicken embryos were similarly pathogenic for 4-day-old turkeys after intratracheal inoculation.

Cryptosporidium spp. have been reported from a wide variety of avian hosts (reviewed by Fayer and Ungar, 1986; Lindsay et al., 1986b; Sundermann et al., 1987b). The parasite completes its life cycle in the microvillous border of epithelial cells in a variety of anatomic sites within these avian hosts (reviewed by Lindsay et al., 1987d). In naturally occurring cryptosporidiosis of birds, respiratory disease (i.e., rales, sneezing, nasal discharge, dyspnea) results when the respiratory epithelium is parasitized (Hoerr et al., 1978; Mason and Hartley, 1980; Dhillon et al., 1981; Tham et al., 1982; Glisson et al., 1984; Itakura et al., 1984; Tarwid et al., 1985; Whittington and Wilson, 1985; Ranck and Hoerr, 1987), and intestinal disease (i.e., diarrhea) results when the small intestine and cecae are parasitized (Slavin, 1955; Hoerr et al., 1986; Ritter et al., 1986; Belton and Powell, 1987).

The significance of cryptosporidial infection limited to the bursa of Fabricius (BF) and cloaca is presently unknown. Most reports do not associate clinical disease with BF only infections (Fletcher et al., 1975; Randall, 1982), however, one study did indicate an association of BF only infections with increased mortality in flocks of chickens suffering from air sac disease due to other pathogens (Gorham et al., 1987). Two reports also have associated cryptosporidiosis with renal disease in birds found dead and examined at necropsy (Gardiner and Imes, 1984; Randall, 1986). The clinical signs of renal cryptosporidiosis are not known.

Current et al. (1986) described *Cryptosporidium baileyi* from broiler chickens and reported that its endogenous life cycle consisted of 3 types of meronts, sexual stages, and unsporulated and sporulated oocysts. The early endogenous stages could be found in the ileum and large intestine, but after 96 hr most stages were confined to the BF and cloaca. *Cryptosporidium baileyi* was differentiated from *C. meleagridis* Slavin, 1955, based on oocyst structure and the site of development of *C. baileyi* in experimentally infected turkeys. Studies in our laboratories with *C. baileyi* have focused on pathogenicity (Lindsay et al., 1986b, 1987a, 1987b, 1987c, 1987d; Blagburn et al., 1987), site specificity (Lindsay and Blagburn, 1986; Lindsay et al., 1986b, 1987b, 1987d), host specificity (Lindsay et al., 1986a, 1987a; Sundermann et al., 1987a), excystation (Sundermann et al., 1987a, 1987b), and chemoprophylaxis of experimental infections (Lindsay et al., 1987c). In none of our studies with *C. baileyi* in chickens or turkeys has diarrhea occurred following oral or intratracheal (IT) inoculation of oocysts. However, respiratory disease does occur following IT inoculation of oocysts (Lindsay et al., 1986b, 1987a, 1987b, 1987c, 1988; Blagburn et al., 1987). We have never observed *C. baileyi* in the small intestines of experimentally infected chickens, turkeys, or ducks, whereas most birds have the infection in the BF and cloaca following oral or IT inoculation of

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* Present address and correspondence: Protozoan Diseases Laboratory, Animal Parasitology Institute, Bldg. 1040, Rm. 102, BARC-East, Beltsville, Maryland 20705.

† Department of Zoology and Wildlife Sciences, Auburn University, Auburn, Alabama 36849.

TABLE I. *Primary avian and mammalian cell cultures and mammalian cell lines examined for their ability to support development of* Cryptosporidium baileyi.

Primary avian cells	Primary mammalian cells	Mammalian cell lines
Fetal chick kidney	Fetal mouse brain	Human fetal lung*—30†
Fetal duck kidney	2-Day-old dog kidney	Fetal mouse brain‡—4
2-Day-old chick kidney	10-Day-old cotton rat kidney	Cotton rat kidney‡—3
2-Day-old duck kidney	10-Day-old cotton rat testicle	Cotton rat testicle‡—3
2-Day-old bobwhite quail kidney	14-Day-old mouse kidney	
16-Day-old chick kidney	14-Day-old mouse testicle	

* Flow 2000, Flow Laboratories, McLean, Virginia.
† Number of times cells had been passaged.
‡ Obtained from primary cell cultures derived in our laboratories.

oocysts. Our studies support the decision of Current et al. (1986) to name *C. baileyi* as a species distinct from *C. meleagridis* which is reported to cause diarrhea in naturally infected birds (turkeys) and to be confined to the terminal ⅓ of the small intestine (Slavin, 1955). The species responsible for causing intestinal cryptosporidiosis in birds (Hoerr et al., 1986; Ritter et al., 1986; Belton and Powell, 1987) other than turkeys is not known, but experimental studies suggest that it is not *C. baileyi.*

The present study was undertaken to further characterize the biology of our isolate (AU-B1) of *C. baileyi* by determining: (1) its ability to develop in primary cell cultures and in cell lines, (2) its host specificity for avian embryos, and (3) the pathogenicity of chicken embryo-passaged oocysts for chickens (10th and 20th passages) and turkeys (10th passage only). Similar studies have been conducted with avian *Eimeria* species (reviewed by Doran, 1982; Speer, 1983) and have added much to our knowledge of these coccidians. Additionally, *C. parvum* from mammals has been successfully grown in cell cultures (Woodmansee and Pohlenz, 1983; Current and Haynes, 1984; Naciri et al., 1986, syn. *C. muris*) and both *C. parvum* (Current and Long, 1983; Naciri et al., 1986) and *C. baileyi* (Current, 1986) will complete development in chicken embryos.

MATERIALS AND METHODS

Preparation of oocysts and sporozoites for inoculations

Oocysts of *C. baileyi* (AU-B1 isolate) were originally isolated from the BF of naturally infected broiler chickens (Lindsay et al., 1986b). Methods of propagation, storage, sterilization, and enumeration for inoculation of oocysts collected from the feces of experimentally infected chickens have been described previously (Lindsay et al., 1986b, 1987a; Sundermann et al., 1987a). Oocysts used for serial passage of *C. baileyi* in chicken embryos were collected in the following manner. Using a Pasteur pipette, the allantoic fluid was removed from each of 8 to 14 specific pathogen-free (SPF) chicken embryos (SPAFAS Inc., Norwich, Connecticut) 8 days postinoculation (PI) of oocysts. Oocysts in the allantoic fluid were concentrated by centrifugation (500 *g* for 10 min) and the supernatant discarded. The pellet containing *C. baileyi* oocysts, erythrocytes, and some cellular debris was resuspended in 0.5% solution of sodium hypochlorite at 4 C for 5 min. This treatment removed most erythrocytes and cellular debris. The sodium hypochlorite was removed by centrifugation (4 times) in sterile Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, New York) and the embryo-passaged oocysts were stored in HBSS containing 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO) (antibiotics) at 4 C until used.

For cell culture studies, sporozoites were obtained from oocysts by incubating oocysts in 0.75% (w/v) sodium taurocholate in HBSS at 40 C for 90–120 min (Sundermann et al., 1987a, 1987b). The sodium taurocholate was removed by centrifugation (500 *g* for 10 min) and the mixture of sporozoites, oocyst walls, and intact oocysts was resuspended in the appropriate volume of cell culture medium or HBSS for inoculations of cell cultures.

Fecal oocysts were stored in HBSS plus antibiotics at 4 C for less than 6 mo for cell culture studies, 3 mo for embryo specificity and initial chicken embryo passage studies, and 2 mo for chicken pathogenicity studies. Embryo-passaged oocysts were stored in HBSS plus antibiotics at 4 C for less than 1 mo for pathogenicity studies. Oocysts were sterilized and counted prior to inoculations as previously described (Lindsay et al., 1986b, 1987a).

Cell cultures

The primary cell cultures and cell lines examined are given in Table I. Cells were grown and maintained in 8-well tissue culture chamber slides (Miles Laboratories, Naperville) using techniques routinely employed in our laboratories (Gargus et al., 1987; Lindsay and Blagburn, 1987). Briefly, the growth media consisted of RPMI-1640 medium with L-glutamine (RPMI) (GIBCO) supplemented with 10–15% (v/v) fetal bovine serum and antibiotics, maintenence media consisted of RPMI-1640 plus 2% fetal bovine serum and antibiotics, and incubation was at 37 C with a 5% CO_2–95% air atmosphere. Cell cultures were inoculated with 2×10^5 sporozoites/well and examined 2, 4, 6, and 8 days PI using an Olympus BH-2 photomicroscope equipped with Nomarski interference-contrast (NIC)

optics to observe living stages. After examination with NIC microscopy, the cell culture slides were stained with Diff-Quick® (Dadge Diagnostic Products, Aguada, Puerto Rico) and examined with bright-field microscopy. Development was recorded as positive only if Type 1 meronts containing 8 merozoites (Current et al., 1986) were observed.

Avian embryo specificity

The ability of *C. baileyi* to complete development in the following avian embryos was examined: turkeys (*Meleagris gallopavo*) (Thaxton Turkeys Inc., Watkinsville, Georgia), domestic ducks (*Anas platyrhynchos*) (SPAFAS Inc.), muscovy ducks (*Cairina moschata*) (Mr. Curtis Day, USDA, Regional Parasite Research Laboratory, Auburn, Alabama), chukar partridges (*Alectoris graeca*), guinea fowl (*Numida meleagris*), ring-necked pheasants (*Phasianas colchicus*) (Stromberg's, Pine River, Minnesota), bobwhite quail (*Colinus virginianus*), and Japanese quail (*Coturnix coturnix*) (G.Q.F. Manufacturing Co., Savannah, Georgia). Four embryos of each species were examined. Embryos were inoculated with 4×10^5 oocysts when 10 days old (chukar partridges, guinea fowl, ring-necked pheasants, bobwhite quail, and Japanese quail) or 14 days old (turkeys, domestic ducks, muscovy ducks). Oocysts were not exposed to excystation solutions because preliminary studies had indicated that oocysts would excyst in the allantoic fluid and produce infections in chicken embryos. Embryos were incubated at 40 C. The chorioallantoic membrane (CAM) was removed from each embryo 6 days PI and examined as a living preparation (Lindsay and Current, 1984) with NIC microscopy for developmental stages of *C. baileyi*.

Pathogenicity of embryo-passaged oocysts

Serial passage of *C. baileyi* was performed by harvesting oocysts from the allantoic fluid of 8–14 infected SPF broiler chicken embryos 8 days PI and inoculating 4×10^5 of these oocysts into the allantoic cavity of each of 8–14 noninoculated 10-day-old SPF broiler chicken embryos. This process was repeated until oocysts that had been passaged 20 times in SPF broiler embryos were obtained.

The pathogenicity of 10th passage (C.b.-10) and 20th passage (C.b.-20) oocysts was evaluated in 2-day-old broiler chickens and 4-day-old turkeys (C.b.-10 only). In experiment I, 6 2-day-old broiler chickens were inoculated IT with 1×10^6 fecal-derived *C. baileyi* oocysts, 6 2-day-old broiler chickens were inoculated IT with 1×10^6 C.b.-10 oocysts, 5 4-day-old turkeys were inoculated IT with 1×10^6 C.b.-10 oocysts, and 2 2-day-old broiler chickens were not inoculated and served as controls. In experiment II, 6 2-day-old broiler chickens were inoculated IT with 1×10^6 fecal-derived *C. baileyi* oocysts, 6 2-day-old broiler chickens were inoculated IT with 1×10^6 C.b.-20 oocysts, and 3 2-day-old broiler chickens were not inoculated and served as controls. All birds in both experiments were housed separately as groups in wire-bottomed cages and given commercial chick starter ration and water *ad libitum*. Birds were observed daily for clinical signs of disease. The feces from each group were examined with NIC microscopy (Lindsay et al., 1986b) on days 4–14 PI for *C. baileyi* oocysts by fecal flotation [illegible] sugar solution. All birds in both experiments were killed by cervical dislocation 14 days PI and examined for gross lesions of airsacculitis due to *C. baileyi* (Lindsay et al., 1986b, 1987a, 1987c, 1988; Blagburn et al., 1987).

RESULTS

Stages that resembled meronts with 4 merozoites were observed in all cell cultures on all days PI. However, on close observation these stages were always found to be sporulated oocysts that had apparently attached to the cells in some manner and not been removed when the monolayers were washed with cell culture medium. No stages containing 8 merozoites were observed in any monolayer; therefore development was considered negative.

All avian embryos examined were positive for development of *C. baileyi* 6 days PI with the exception of a single chukar partridge embryo. Gross lesions were not observed on the CAM of any embryo and no deaths occurred during the study. The majority of stages observed with NIC microscopy were asexual stages and macrogamonts; microgamonts and unsporulated and sporulated oocysts were present in each positive embryo but were fewer in number than asexual stages and macrogamonts. The merozoites were motile and moved vigorously in a circular pattern within the parasitophorous vacuole. This movement usually preceded release of the merozoites from the meront onto the endodermal surface of the CAM where they glided in a rapid serpentine or semicircular fashion. Both thick- and thin-walled oocysts were observed to excyst and release their sporozoites onto the endodermal surface of the CAM. These sporozoites were motile and underwent movements similar to those of merozoites.

All inoculated birds in both experiments developed clinical signs of respiratory tract disease beginning 5 days PI and continuing until the experiment was terminated on day 14 PI. Clinical signs consisted of moist rales, dyspnea, sneezing, and reluctance to move. Clinical signs were not observed in noninoculated controls. One chicken in experiment I inoculated with fecal-derived oocysts died 12 days PI and 2 turkeys given C.b.-10 oocysts died on days 9 and 14 PI. One chicken inoculated with fecal-derived oocysts died 10 days PI in experiment II. No control chickens died during the study. Gross lesions of airsacculitis consisting of variable accumulations of white, foamy fluid within the air sacs were observed in all sacrificed birds that were inoculated with either fecal-derived oocysts, C.b.-10 oocysts, or C.b.-

20 oocysts. All birds that died also had similar gross lesions of airsacculitis. Gross lesions were not observed in controls. Oocysts were detected in the feces of all inoculated groups 5–14 days PI. Oocysts were not observed in the feces of control chickens.

DISCUSSION

Current and Haynes (1984) reported complete development of *C. parvum* in human fetal lung, primary chicken kidney, and porcine kidney cell cultures and Naciri et al. (1986) also obtained complete development of *C. parvum* in baby hamster kidney cell cultures. Woodmansee and Pohlenz (1983) found mature and developing asexual stages (number of types or generations not given) of *C. parvum* in human rectal tumor cell cultures. We observed no development of *C. baileyi* in any of the 12 primary cell cultures or 4 cell lines examined in our study. These differences may be due to inherent differences in the ability of *C. parvum* and *C. baileyi* to develop in cell cultures in a similar manner as different species of *Eimeria* differ in their abilities to grow in cell cultures (Doran, 1982; Speer, 1983). It is unlikely that our inability to demonstrate development of *C. baileyi* in cell cultures was a result of the choice of cells because we used 6 types of primary avian cell cultures, and primary cell cultures from the natural host usually support the most development of *Eimeria* species *in vitro* (Doran, 1982). Additionally, we examined human fetal lung cells that were shown to support complete development of *C. parvum* (Current and Haynes, 1984). As in the present study, Woodmansee and Pohlenz (1983) found that oocysts often adhered to cultured cells and made differentiation of developmental stages from inoculated oocysts difficult. Because 8 merozoites are present in the Type 1 meronts of *C. baileyi* (Current et al., 1986), we attempted to identify this stage because it could not be confused with inoculated oocysts.

Chicken embryos do not support the development of all *Eimeria* species of chickens (Doran, 1982) and the species that do develop in chicken embryos can seldom develop in embryos of other avian species (Doran, 1982; Kogut et al., 1983). This host embryo specificity can be overcome somewhat by immunosuppression of recipient embryos or by using oocysts that have already adapted to chicken embryos as the source of sporozoites for recipient nonhost embryos (Doran, 1982; Kogut et al., 1983). In the present study, *C. baileyi* readily underwent complete development in all 8 species of avian embryos examined without prior manipulation of the embryos or *C. baileyi* oocysts. Therefore, the present study suggests that *C. baileyi* does not exhibit host specificity for avian embryos. The absence of infection in the single chukar partridge embryo was probably due to an error in inoculation because the other 3 chukar partridge embryos were infected with *C. baileyi*. Interestingly, we were able to infect bobwhite quail embryos in the present study, but our attempts (Lindsay et al., 1986a) and those by others (Current et al., 1986) to infect bobwhite quail by oral inoculation of *C. baileyi* oocysts have proven unsuccessful. This further indicates a lack of host embryo specificity of *C. baileyi*.

Serial passage of chicken *Eimeria* spp. in chicken embryos has been widely used to develop embryo-adapted strains (Doran, 1982). With successive passages, the numbers of oocysts produced in the embryos increases as the parasite becomes adapted to embryonic development, and the parasites become less pathogenic for the embryo and the chicken host (Doran, 1982). We did not monitor oocyst production in our chicken embryos infected with *C. baileyi* and therefore refer to oocysts only as embryo passaged.

Current and Long (1983) reported infections in mice with *C. parvum* oocysts collected from chicken embryos, however they were not able to obtain enough oocysts for serial passage of *C. parvum* in chicken embryos. Naciri et al. (1986) were able to obtain enough *C. parvum* oocysts and other infective stages from chicken embryos and passaged the parasite 45 times in chicken embryos. They reported that after the 45th passage the numbers of oocysts produced in the allantoic fluid decreased and that most stages present were sporozoites and merozoites. Qualitatively, we noticed no such decrease in oocyst production during the 20 times we passaged *C. baileyi* in chicken embryos. It is unclear if Current (1986) passaged *C. baileyi* in avian embryos. His report only mentions using chicken embryos to obtain oocysts for the preparation of antigens for immunological studies.

In our study, *C. baileyi* retained its pathogenicity for 2-day-old broiler chickens after 10 and 20 passages in chicken embryos. This was evidenced by the production of clinical signs of respiratory disease and gross lesions of airsacculitis in IT-inoculated birds that were indistinguishable from those in 2-day-old broiler chickens

given equal numbers of fecal-derived oocysts. Although the absence of mortality in chickens inoculated with C.b.-10 or C.b.-20 oocysts might suggest that there was some minimal decrease in pathogenicity, too few chickens were examined to make definitive statements about reductions in mortality. The C.b.-10 oocysts were still highly pathogenic for 4-day-old turkeys, causing clinical respiratory disease, airsacculitis, and mortality following IT inoculation. The clinical signs and gross lesions seen in turkeys inoculated with C.b.-10 oocysts were similar to those seen in turkeys inoculated IT with 1×10^6 fecal-derived oocysts of *C. baileyi* (Lindsay et al., 1987a), which indicates that there was no reduction in pathogenicity for turkeys following 10 passages in chicken embryos. The prepatent period in chickens and turkeys was not influenced by passage in chicken embryos because all inoculated groups passed oocysts 5 days PI which is normal for the AU-B1 isolate of *C. baileyi* (Lindsay et al., 1986b, 1988). Because all birds were killed 14 days PI the influence of embryo passage on the patent period could not be evaluated.

No anticoccidial drugs tested *in vivo* have prevented clinical respiratory cryptosporidiosis in chickens (Lindsay et al., 1987c). The lack of development of *C. baileyi* in cell cultures makes chicken embryos an attractive alternative to *in vivo* drug studies, since chicken embryos have been used to test anticoccidial drugs against *Eimeria* spp. in the past (Doran, 1982). However, the lack of gross lesions and deaths resulting from *C. baileyi* infections in chicken embryos would necessitate an all-or-none drug screen, or statistical analysis of differences in the numbers of oocysts produced in the allantoic fluid of infected embryos.

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THE DEVELOPMENT OF *EUSTRONGYLIDES TUBIFEX* (NEMATODA: DIOCTOPHYMATOIDEA) IN OLIGOCHAETES

Lena N. Measures
Department of Zoology, College of Biological Science, University of Guelph,
Guelph, Ontario, Canada N1G 2W1

ABSTRACT: Egg development of *Eustrongylides tubifex* (Nitzsch *in* Rudolphi, 1819) Jägerskiöld, 1909, was studied at 5, 10, 15, 20, 25, and 30 C. Eggs developed at 20, 25, and 30 C. Development ceased at 0, 5, 10, and 15 C but resumed when temperatures were raised. Eggs contained first-stage larvae in 23–26 days at 25 C. Seven species of aquatic oligochaetes were exposed experimentally to eggs of *E. tubifex* containing first-stage larvae. Third-stage larvae of *E. tubifex* developed in the aquatic oligochaetes, *Limnodrilus hoffmeisteri* Claparède, 1862, and *Tubifex tubifex* (Müller, 1774). Larvae developed in the ventral blood vessel of oligochaetes. Second-stage larvae were seen first in oligochaetes 30 days postinfection and third-stage larvae were seen 70–109 days postinfection at 25 C. Third-stage larvae in oligochaetes retained the cuticle of the first and second molt.

Transmission of *Eustrongylides* spp. (Nematoda: Dioctophymatoidea) involves an aquatic oligochaete first intermediate host and a fish second intermediate host; fish may also serve as paratenic hosts by means of piscivory (Karmanova, 1968). Tubificids (*Limnodrilus* sp., *Tubifex tubifex* (Müller, 1774)) and *Lumbriculus variegatus* (Müller, 1774) were implicated by Karmanova (1965, 1968) as first intermediate hosts of *Eustrongylides excisus* Jägerskiöld, 1909 in an enzootic area in the Volga Delta, U.S.S.R. Hirshfield et al. (1983) suggested that *Limnodrilus hoffmeisteri* Claparède, 1862 may be an intermediate host of *Eustrongylides* sp. in an enzootic area in Maryland where the prevalence of larval *Eustrongylides* sp. in wild mummichog (*Fundulus heteroclitus* (Linnaeus)) was high and where *L. hoffmeisteri* was abundant. Lichtenfels and Stroup (1985) reported 1 naturally infected aquatic oligochaete identified as a tubificid from the Chesapeake Bay area. The oligochaete was immature sexually, damaged, and could not be identified beyond the family level (Tubificidae).

The present study shows that third-stage larvae of *Eustrongylides tubifex* (Nitzsch *in* Rudolphi, 1819) Jägerskiöld, 1909 develop in tubificids (*Limnodrilus hoffmeisteri, Tubifex tubifex*). Development of eggs of *E. tubifex* at various temperatures and development of larvae of *E. tubifex* in aquatic oligochaetes in the laboratory are described.

MATERIALS AND METHODS

Culture of eggs of *E. tubifex*

Eggs dissected from the uterus of gravid *E. tubifex* from wild or experimentally infected mergansers (*Mergus merganser* L. and *M. serrator* L.) were used to study development of eggs at 22 and 25 C. Eggs were washed in avian saline and placed in petri dishes (35 × 10 mm) containing distilled water. Formalin (0.1%) or cycloheximide (BDH Chemicals, 350 Evans Avenue, Toronto, Ontario) (25 mg in 500 ml water) were added to reduce fungal growth. Eggs were removed periodically from petri dishes, fixed in hot glycerin alcohol (1 part glycerin : 9 parts 70% alcohol), cleared in glycerin, and development was studied microscopically.

Effect of temperature on egg development

Eggs dissected from the posterior extremity of the uterus of gravid *E. tubifex* from an experimentally infected red-breasted merganser (*Mergus serrator*) were used to study development of eggs at 5, 10, 15, 20, and 25 C and to determine the optimum temperature for development. Eggs were pipetted into 25 petri dishes (35 × 10 mm) containing distilled water. Five petri dishes, each containing approximately 100–1,000 eggs, were placed in incubators set at each of the above 5 temperatures. Times required for eggs to develop and contain first-stage larvae were noted. The number of eggs containing larvae was expressed as a ratio of the total number of eggs. Results were analyzed using single factor analysis of variance after arcsin transformation of data. The least significant difference test was performed to locate significant temperature differences (Sokal and Rohlf, 1981; Zar, 1984).

First-stage larvae were removed from eggs as follows. Eggs containing larvae were placed in a 1:1 solution of 3% sodium hydroxide and 3% sodium hypochlorite for 24 hr at 22 C. They were washed 4 times in piscine saline by centrifuging the solution 1 min at 1,000 rpm then removing the supernatant and adding fresh saline. Larvae were then liberated from eggs by centrifugation in saline 12 min at 2,500 rpm (Elliott, 1954).

Maintenance and experimental infection of aquatic oligochaetes

Aquatic oligochaetes were collected from the Keating Channel, Toronto harbor (43°38′N, 79°22′W) using an Ekman grab. This site was chosen as tubificids were abundant there and *Limnodrilus hoffmeisteri*, a suspected oligochaete intermediate host, was reported to dominate the oligochaete assemblage in the harbor (Brinkhurst, 1970). Bottom sediment was transported

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to the laboratory and placed in aerated aquaria at room temperature (20–23 C). *Lumbriculus variegatus* (Müller, 1774) was collected from a small pond on Eight-Mile Point, Lake Simcoe, Ontario (44°31′N, 79°25′W) following methods of Mace and Anderson (1975). Other oligochaetes were collected from streams in the Guelph area.

Eggs (20–50) containing larvae of *E. tubifex* were pipetted into petri dishes (35 × 10 mm) containing aquaria water and detritus. In each of these dishes 1 aquatic oligochaete was allowed to feed on the detritus and eggs for 24–48 hr. Oligochaetes were then transferred to fresh water and cleaned of detritus and any remaining eggs with the aid of fine brushes. Oligochaetes were placed in small (2-L) aerated aquaria containing sand, detritus, decomposing leaves, and water at 27.5 ± 2.4 C. Oligochaetes were anaesthetized later in carbonated water, dissected in 0.65% saline, and examined for larvae of *E. tubifex*. Some oligochaetes containing larvae were fixed in 10% buffered formalin, dehydrated, embedded, sectioned, and stained by standard histologic procedures. Oligochaetes were identified and some were sent to Dr. R. O. Brinkhurst, Institute of Ocean Sciences, Sidney, B.C. for confirmation. Nomenclature of oligochaetes follows Brinkhurst (1986).

Preservation of larvae

Larvae dissected from oligochaetes were fixed in hot glycerin alcohol. Worms were cleared by allowing the alcohol to evaporate. Cleared worms were studied, measured, and drawn using a compound microscope equipped with a drawing tube or a dissecting microscope equipped with a camera lucida. The mean and range of measurements of larvae are given except where indicated otherwise. Some larvae were prepared for scanning electron microscopy by fixing in hot 10% buffered formalin, postfixing in 1% osmium tetroxide at 4 C for 2 hr, and dehydrating in a graded series of alcohols. Worms in absolute alcohol were then dried in a critical point dryer by carbon dioxide substitution. Worms were coated with gold-palladium and examined using a JEOL JSM-35C scanning electron microscope.

RESULTS

Morphology and development of eggs at room temperature

Eggs from the posterior part of the uterus of *E. tubifex* from wild common mergansers (*Mergus merganser*) measured 36 (34–37) μm wide and 70 (66–75) μm long (n = 10). These eggs contained 1 cell and were amber with a thick, pitted shell. Polar regions of the egg shell appeared relatively transparent compared with the rest of the egg shell. These eggs contained 2 cells 1–2 days after incubation in distilled water at 22 C (Fig. 1). After 12 days at 22 C a space developed between the embryo and the egg shell at 1 pole. This space enlarged around the embryo and the embryo became indented on 1 side. Eggs contained motile first-stage larvae 31–33 days at 22 C and 23 days at 25 C (Fig. 2). These first-stage larvae moved slightly and infrequently for 1 wk. Eggs from the anterior part of the uterus were single-celled and white with a thin, pitted shell and did not develop in distilled water at 25 C.

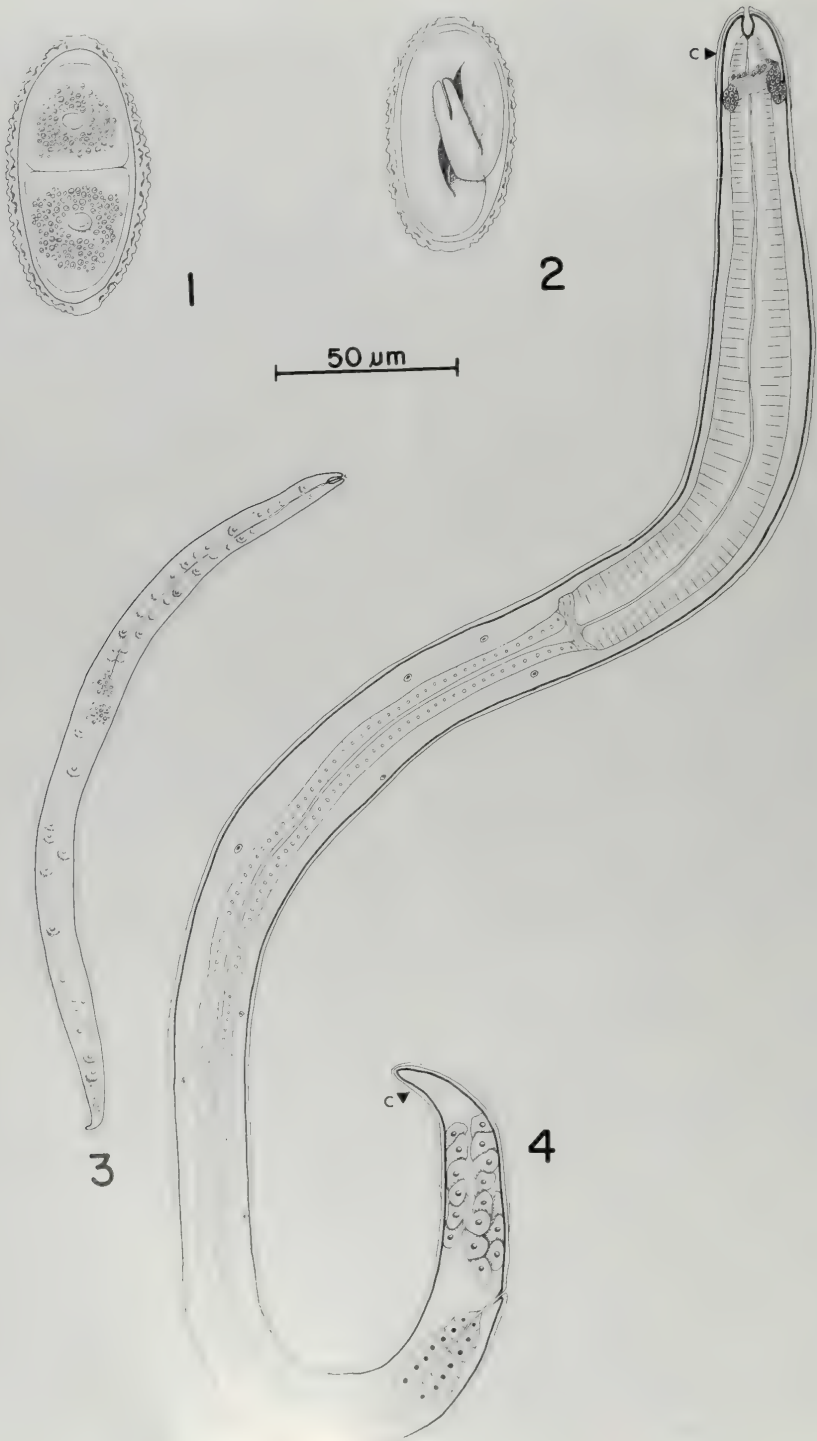
Development of eggs at various temperatures

Eggs from the posterior part of the uterus of gravid *E. tubifex* from an experimentally infected red-breasted merganser were used to determine whether they would develop at 5, 10, 15, 20, and 25 C. Eggs kept at 25 and 20 C contained larvae in 23–26 and 36–42 days, respectively (Table I). There was no significant difference ($P < 0.05$) in the proportion of eggs containing larvae at 25 and those at 20 C. Eggs kept at 5 and 10 C remained in the 2-cell stage for 101 days. Slight development of eggs (multiple cleavage and enlargement of the polar space) occurred at 15 C in 101 days. Some eggs kept at 5, 10, and 15 C developed to first-stage larvae after being transferred to 25 C by weekly increments of 5 C (Table I). There were no significant differences ($P < 0.05$) between the 3 groups in the proportion of eggs containing larvae when transferred to 25 C after originally being kept at 5, 10, or 15 C. The proportion of eggs containing larvae in each of the latter 3 transferred groups was significantly less than the proportion of eggs maintained continually at 25 C ($P > 0.05$). In a preliminary experiment, eggs also developed at 30 C. Eggs kept at 0 C for 88 days developed normally when transferred to 20 C.

Eggs containing first-stage larvae stored in distilled water containing cycloheximide at 4 C remained infective to aquatic oligochaetes for at least 2½ yr.

Experimental infection of oligochaetes

Over 1,000 aquatic oligochaetes of 7 species were exposed experimentally to infective eggs of *Eustrongylides tubifex* (Table II). Over 100 control oligochaetes from the Keating Channel were examined and none were infected. Most oligochaetes were not mature sexually and could not be identified to species. Many oligochaetes exposed to eggs failed to become infected and many died from unknown causes during the course of the experiment. Nevertheless, 44 oligochaetes became infected and larval *E. tubifex* developed in adult *Limnodrilus hoffmeisteri* and *Tubifex tubifex*. Oligochaetes (n = 104) were examined at intervals from 2 to 25 days postinfection (PI) and none was infected. However, larvae were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seen first in oligochaetes at 30 days PI. Larvae from 30 to 109 days PI were in the ventral blood vessel of oligochaetes from segment II to the segment anterior to the anus. There were no apparent histologic changes in infected oligochaetes (Figs. 5–9). Larvae at 30 days PI were second stage, immobile, and occurred singly or in groups within the ventral blood vessel, particularly near the gonads (segments X–XI) (Table III). Twenty-eight second-stage larvae were found in 1 *Limnodrilus* sp. 30 days PI. Larvae were in the third-stage as early as 70 days PI. Third-stage larvae retained the cuticle of the first and second stage.

The frequency distribution of infected oligochaetes indicated that few oligochaetes had more than 3 larvae (Table IV). Fourteen second-stage larvae in 1 *Limnodrilus* at 52 days PI were significantly smaller ($P < 0.05$, Student's *t*-test) (1.16 [range of 0.74–1.45] mm long) than 7 second-stage larvae from another *Limnodrilus* sp. (2.23 [1.02–3.40] mm long). Four larvae were recovered from 1 oligochaete at 102 days PI (Table III). Reproductive primordia were poorly developed in third-stage larvae in oligochaetes with 2 or more larvae compared with larvae in oligochaetes with single infections. In addition these larvae were usually shorter in length. One early third-stage larva 8.2 mm long and 2 second-stage larvae 1.6 and 2.2 mm long were recovered from an oligochaete 102 days PI. Four third-stage larvae were similar in length (11.1, 10.7, 9.6, 11.2 mm) in another oligochaete 102 days PI. The degree of development of the reproductive primordia in each of these 4 third-stage larvae was similar to each other. The intensity of larvae in experimentally infected oligochaetes was 1.3 (1–2) in *T. tubifex* and *Tubifex* sp., 5.5 (2–9) in *Limnodrilus hoffmeisteri*, and 4.8 (1–28) in *Limnodrilus* sp.

Description of first-stage larva
(Fig. 3)

First-stage larvae from eggs (n = 10) 236 (208–252) μm long, maximum width midbody 12 (11–14) μm. Larvae slender with delicate, smooth cuticle and tapered tail. Buccal cavity cylindrical. Stylet 14 (11–18) μm long. Oesophagus 87 (72–98) μm long, poorly defined, consisting of loose aggregate of cells with conspicuous lumen. Intestine not developed. Posterior extremity of larva containing scattered cells with prominent nuclei. Genital primordium and nerve ring not observed.

TABLE I. *Number of eggs containing larvae of* Eustrongylides tubifex *incubated at various temperatures.*

Temperature (C)	Days incubated	Mean percent of eggs* ± SD (range) containing larvae
25	23–26	44.4 ± 8 (38–58)
20	36–42	61.4 ± 34 (5–91)
15	101†	0 (put at 25 C, see A)‡
10	101†	0 (put at 25 C, see B)
5	101†	0 (put at 25 C, see C)
25	A) 14 days	16.5 ± 13 (5–39)
	B) 22 days	21.9 ± 8 (11–30)
	C) 22 days	19.0 ± 15 (6–44)

* Five replicates at each temperature.

† Eggs incubated at 15, 10, and 5 C for 101 days were transferred to 25 C by weekly increments of 5 C.

‡ A, B, and C represent times required for eggs to contain larvae after being placed at 25 C.

Description of second-stage larva
(Figs. 4, 10–15)

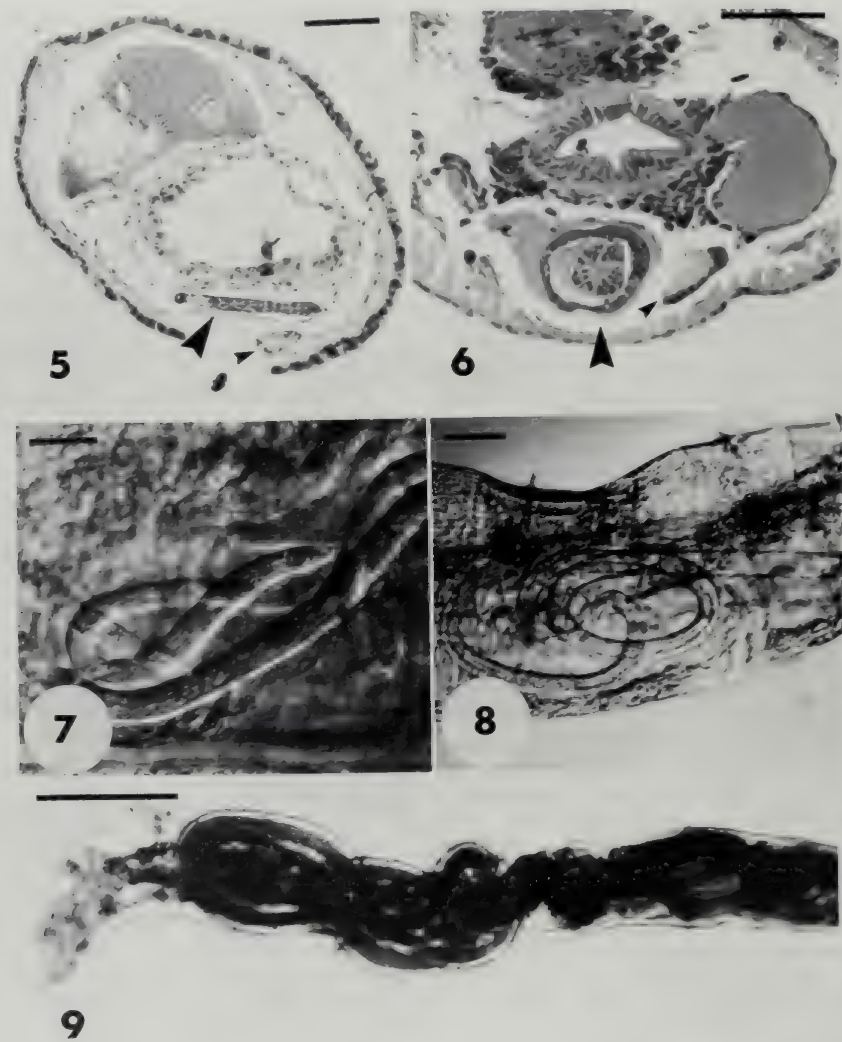
First molt occurred less than 30 days PI in oligochaetes. Cuticle of first-stage not shed; visible at the extremities.

Thirty days PI: Second-stage larvae (n = 10) first observed 30 days PI in *Limnodrilus* sp., 588 (398–700) μm long and 24 (17–28) μm wide at oesophageal–intestinal junction. Larvae slender with thin, smooth cuticle. Buccal cavity prominent, 8 (7–13) μm long. Stylet not observed. Cuticle and hypodermis around oral opening slightly elevated. Nerve ring 20 (13–29) μm from anterior extremity. Oesophagus well developed, 221 (164–290) μm long. Oesophageal lumen present. Oesophageal–intestinal sphincter not pronounced. Intestine consisting of cells with prominent nuclei. Intestinal lumen present. Rectum 20 (11–26) μm long, tapered from intestine to anus. Tail 76 (37–105) μm long, less tapered than in first stage.

Thirty-seven days PI: Two second-stage larvae recovered from *Limnodrilus hoffmeisteri* 37 days PI; 788 μm long and 38 μm wide at oesophageal–intestinal junction. Oesophagus and intes-

←

FIGURES 1–4. Eggs and larvae of *Eustrongylides tubifex*. **1.** Egg containing 2 cells at 3 days at 20 C. **2.** Egg containing first-stage larva at 32 days (note stylet) at 25 C. **3.** First-stage larva removed from egg (note stylet). **4.** Second-stage larva from *Limnodrilus* sp. 30 days postinfection, left lateral view (note: the first-stage cuticle [c] is retained, visible at cephalic and caudal extremities).



FIGURES 5–9. Aquatic oligochaetes experimentally infected with *Eustrongylides tubifex* incubated at 25 C. **5.** Histologic cross section of *Limnodrilus hoffmeisteri* 30 days postinfection (note: second-stage larva cut longitudinally in ventral blood vessel [large arrow]; ventral nerve cord denoted by small arrow). H&E. Scale bar = 70 μm. **6.** Histologic cross section of *Limnodrilus* sp. 80 days postinfection (note: third-stage larva in ventral blood vessel [large arrow]; ventral nerve cord denoted by small arrow). H&E. Scale bar = 70 μm. **7.** Four second-stage larvae in ventral blood vessel of *Limnodrilus* sp. 30 days postinfection. Scale bar = 50 μm. **8.** Three second-stage larvae in ventral blood vessel of *Limnodrilus* sp. 52 days postinfection. Scale bar = 150 μm. **9.** One third-stage larva in ventral blood vessel of *Limnodrilus* sp. 85 days postinfection. Scale bar = 210 μm.

tine well developed, attached to body wall by minute branched processes. Cephalic extremity rounded bluntly and cuticle and hypodermis around oral opening elevated. Tapered tail 78–86 μm long and granular in appearance.

Fifty-two to 56 days PI: Second-stage larvae recovered from *Limnodrilus* sp. 52 days PI; 1.56 (0.74–3.40) (n = 23) mm long and 49 (35–62) (n = 10) μm wide at oesophageal–intestinal junction. Cephalic extremity rounded bluntly, cuticle

TABLE II. *Aquatic oligochaetes exposed experimentally to eggs of* Eustrongylides tubifex.

Species	n	Number infected (percent of n)
Limnodrilus cervix	10	0
Limnodrilus hoffmeisteri	83	4 (5)
Limnodrilus sp.*	548	37 (7)
Tubifex tubifex	26	2 (8)
Tubifex sp.*	57	1 (2)
Ilyodrilus templetoni	4	0
Dero digitata	5	0
Lumbriculus variegatus	74	0
Sparganophilus tamesis	1	0
Total	808	44 (5)

* Immature.

and hypodermis around oral opening prominent. Buccal cavity 15 (10–24) (n = 10) μm long. Oesophagus 751 (302–1,206) (n = 10) μm long. Nerve ring 48 (32–68) (n = 10) μm from anterior extremity. Rectum 77 (36–100) (n = 10) μm long. Space developing between body wall and cuticle of posterior extremity, indicating that resorption of tail occurring. Tail 123 (80–166) (n = 10) μm long. Three to 4 prominent cells near distal extremity of the rectum.

Two second-stage larvae recovered from *Limnodrilus* sp. 55 and 56 days PI; 5.1–5.6 mm long and 70–95 μm wide at oesophageal–intestinal junction. These second-stage larvae considerably larger than those at 52 days PI. Tail short (25–38 μm long) and body wall withdrawn anteriorly toward anus leaving transparent tail. Resorption of tail also greater than in larvae at 52 days PI.

Seventy to 80 days PI: Five second-stage larvae recovered from *Limnodrilus* sp. and *Tubifex tubifex* 70–80 days PI; 2.7–4.9 mm long and 49–95 μm wide at oesophageal–intestinal junction. Cephalic extremity rounded bluntly. Tail 69–121 μm long.

Description of third-stage larvae
(Figs. 16–21; Table V)

Second molt occurred 70–109 days PI. Live larvae bright red. Cuticle of second stage not shed. Thus, 3 cuticles visible at extremities, namely, outermost first-stage cuticle, middle second-stage cuticle, and innermost newly formed third-stage cuticle.

Cuticle of third-stage larvae recovered from *Limnodrilus* sp., *Tubifex tubifex,* and *Tubifex* sp. 70–109 days PI with fine longitudinal striations except in 4 unstriated bands extending from cephalic to posterior extremity. Cephalic extremity

TABLE III. *Number and stage of larvae of* Eustrongylides tubifex *recovered from experimentally infected oligochaetes incubated at 25 C.*

Species (n)		Days PI	Number and stage of larvae*
Tubifex sp.	(1)	70	1 L3
T. tubifex	(1)	70	1 L2
T. tubifex	(1)	109	2 L3
Limnodrilus hoffmeisteri	(1)	30	8 L2
Limnodrilus hoffmeisteri	(1)	30	9 L2
Limnodrilus hoffmeisteri	(1)	30	3 L2
Limnodrilus hoffmeisteri	(1)	37	2 L2
Limnodrilus sp.	(12)	30	10 (1–28) L2
Limnodrilus sp.	(3)	52	9 (2–18) L2
Limnodrilus sp.	(1)	55	2 L2
Limnodrilus sp.	(1)	56	1 L2
Limnodrilus sp.	(3)	80	1 (1–2) L2
Limnodrilus sp.	(1)	82	1 L3
Limnodrilus sp.	(1)	85	1 L3
Limnodrilus sp.	(1)	91	1 L3
Limnodrilus sp.	(1)	100	1 L3
Limnodrilus sp.	(8)	102	2 L2, 2 (1–4) L3
Limnodrilus sp.	(5)	109	1 (1–2) L3
Total	(44)		

* Number of larvae are mean with range in parentheses. L2—second-stage larvae. L3—third-stage larvae.

conical with 12 labial papillae in 2 circles, each with 6 papillae. Two lateral, 2 subdorsal, and 2 subventral inner circle labial papillae with narrow bases and spike-like apices. Two lateral, 2 subdorsal, and 2 subventral outer circle labial papillae with broad bases and nipple-like apices. Outer lateral labial papillae double. Four small lateral field papillae present between inner and outer circle of labial papillae. Amphids and ventral papilla not seen. Somatic papillae present laterally. Suspensory muscles from intestine to body wall well developed. No significant difference in total length ($P < 0.05$, Student's t-test) of male and female larvae (Table V). Anus terminal and posterior extremity blunt; in males posterior extremity contracting slightly inward at anus. Vagina developed partially with thick wall. Genital primordium extending anteriorly short distance from patent, ventro-terminal vulva, then curving posteriorly and terminating close to body wall near posterior end of intestine. Male genital primordium extending ventrally from rectum short distance anterior to posterior end of rectum and terminating in blunt extremity. Spicule primordium developed poorly, extending dorsally from posterior end of rectum almost to oesophageal–intestinal junction. Intestinal lumen narrow. Three papillae present dorsal to anus. Voucher specimens (USNM Helm. Coll. No. 79994) deposited in National Parasite Collection, Beltsville, Maryland, U.S.A.

TABLE IV. *Frequency distribution of* Eustrongylides tubifex *larvae in experimentally infected aquatic oligochaetes 30–109 days postinfection incubated at 25 C.*

	Number of larvae*													
	1	2	3	4	5	7	8	9	11	13	16	17	18	28
Number of infected oligochaetes (n = 44)	16	9	5	2	1	1	3	1	1	1	1	1	1	1

* Infected oligochaetes were *Limnodrilus hoffmeisteri*, *Limnodrilus* sp., *Tubifex tubifex*, and *Tubifex* sp. Those oligochaetes with 9 or more larvae were from infections 30 and 52 days postinfection.

DISCUSSION

Adult female *E. tubifex* oviposited eggs with shells whether or not females were fertilized by males. Two pronuclei were seen in some 1-celled eggs indicating that sperm had penetrated the egg but syngamy had not yet occurred. Malakhov and Spiridonov (1983) also observed 2 pronuclei in eggs of *E. excisus* prior to the first cleavage. Karmanova (1968) also reported that 1-celled eggs from the uterus of *E. excisus* underwent cleavage, producing 2 blastomeres, by the third to fourth day. These findings differ from those of Mace and Anderson (1975), who indicated that only 2-celled eggs from the uterus of *Dioctophyma renale* were fertilized and developed. They did observe 1-celled eggs from the uterus of adult female *D. renale* which were fertilized. However, these 1-celled eggs did not develop.

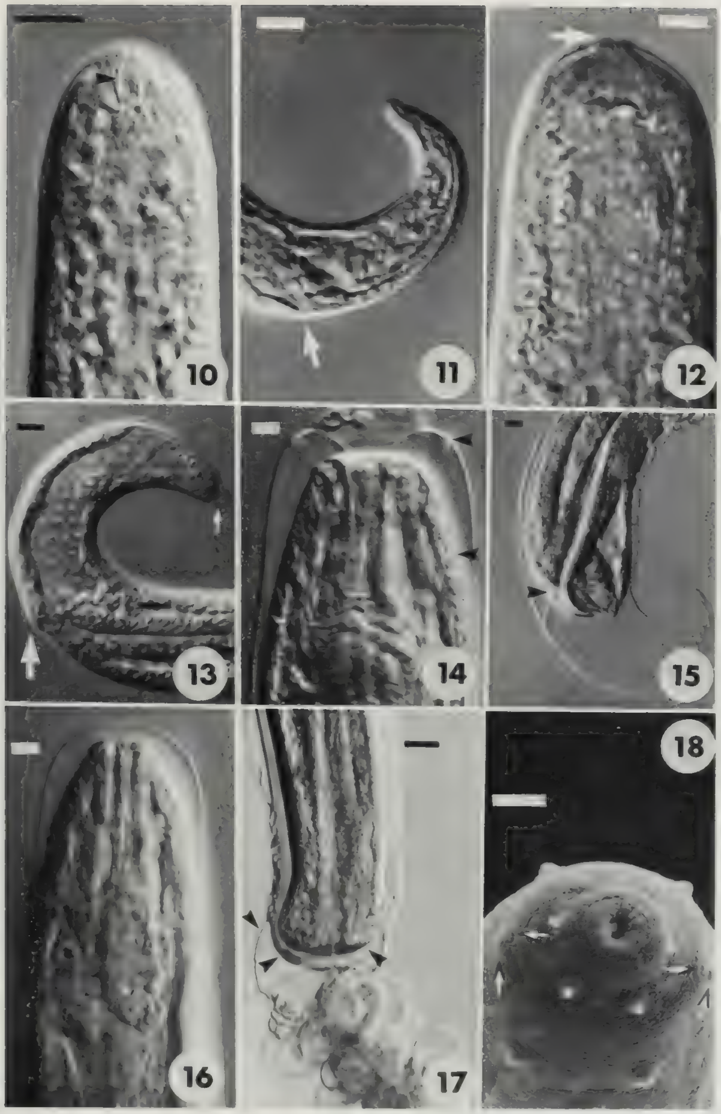
Temperature experiments indicated that eggs of *E. tubifex* can survive a wide range of environmental temperatures. This was also observed in *E. excisus* by Karmanova (1968) and in *D. renale* by Mace and Anderson (1975). In addition, the latter authors also reported that development ceased at lower temperatures but resumed when temperatures were raised.

In the present study, *Eustrongylides tubifex* developed to the third stage in experimentally infected aquatic oligochaetes, *Limnodrilus hoffmeisteri* and *Tubifex tubifex*. Eggs containing first-stage larvae were egested by *L. variegatus* (cf. Karmanova, 1965, 1968), the only known intermediate host of *Dioctophyma renale* (Karmanova, 1959, 1960, 1962; Mace and Anderson, 1975).

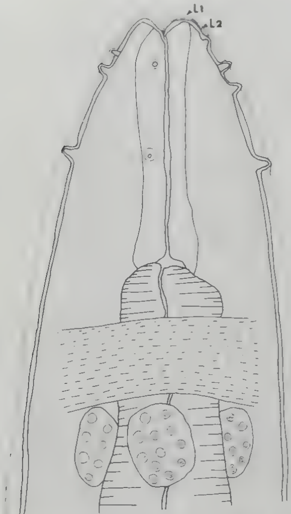
Although as many as 28 second-stage larvae of *E. tubifex* were present in 1 oligochaete at 30 days PI, 4 was the maximum number of third-stage larvae observed in any 1 oligochaete. The size and development of larvae were influenced by the total number of larvae present. For example, as early as 52 days PI, 14 second-stage larvae present in 1 *Limnodrilus* sp. were significantly smaller than 7 second-stage larvae in another *Limnodrilus* sp. Karmanova (1965, 1968) also observed larvae of *E. excisus* in the ventral blood vessel of oligochaetes and noted that development was slow and asychronous when several larvae were present. Mace and Anderson (1975) reported similar findings in *D. renale* larvae in *L. variegatus*.

The present study suggests that during the early course of infection, larvae of *E. tubifex* may be found anywhere in the ventral blood vessel. As early as 30 days PI, larvae apparently moved to the anterior region of the oligochaete, particularly to the region near the gonads (segments X–XI), and late second-stage and third-stage larvae were always found in this region. It is not known why larvae migrate to this anterior location, although blood vessels are larger there. The dorsal blood vessel may be unsuitable for developing larvae because of the presence of valves and peristalsis. In oligochaetes the dorsal blood vessel transports blood anteriorly into the ventral blood vessel via lateral segmental vessels and anterior commissural vessels (commonly termed pseudo-hearts). These pseudo-hearts and the dorsal blood vessel are contractile and possess 1-way valves (Stephenson, 1930; Brinkhurst and Jamieson, 1971). As mentioned earlier, *Dioctophyma renale* develops in the aquatic oligochaete, *Lumbriculus variegatus*. The dorsal blood vessel of *L. variegatus* lacks valves although another kind of occluding apparatus (muscular fibers) is present (Stephenson, 1930), which might prevent larvae of *D. renale* from developing in this region of the circulatory system.

Morphology of first-stage larvae of *E. tubifex* was similar to that of *E. excisus* described by Karmanova (1965, 1968) except that 2 cells, one described as excretory and the other described as the genital primordium, were not seen. First-stage larvae of *E. tubifex* (208–252 μm) were similar in length to those reported in *E. excisus* (240–298 μm) by Karmanova (1965); later, how-



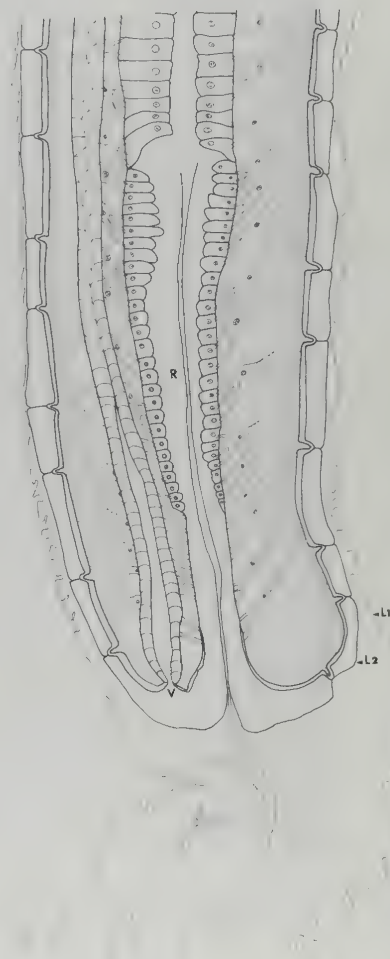
FIGURES 10–18. Second- and third-stage larval *Eustrongylides tubifex* from experimentally infected aquatic oligochaetes incubated at 25 C. Scale bar = 10 μm. **10.** Cephalic extremity of second-stage larva from *Limnodrilus hoffmeisteri* 30 days postinfection (note: buccal cavity [arrow]). **11.** Caudal extremity of second-stage larva from *Limnodrilus hoffmeisteri* 37 days postinfection. Arrow denotes anus (note: tail contains many cells). **12.** Cephalic extremity of second-stage larva from *Limnodrilus* sp. 52 days postinfection. Arrow denotes raised cuticle and hypodermis near mouth. **13.** Caudal extremity of second-stage larva from *Limnodrilus* sp. 52 days postinfection (note: resorption of cells occurring in tail). Large arrow denotes anus. Loose first-stage cuticle is visible at tip of tail (small arrow). **14.** Cephalic extremity of second-stage larva from *Limnodrilus* sp. 56 days postinfection (note: loose outer first-stage cuticle and inner second-stage cuticle [arrows]; also note blunt anterior extremity). **15.** Caudal extremity of second-stage larva from *Limnodrilus* sp. 56 days postinfection (note: tail is almost completely resorbed). Arrow denotes anus. **16.** Cephalic extremity of third-stage larva from *Limnodrilus* sp. 70 days postinfection (note: conical anterior extremity). **17.** Caudal extremity of third-stage larva from *Limnodrilus* sp. 82 days postinfection (note: loose outer first-stage and inner second- and third-stage cuticles [arrows]). **18.** Scanning electron micrograph of cephalic extremity of third-stage larva from *Limnodrilus* sp. 109 days postinfection (note: lateral outer labial papillae are double [arrows]).



19

50 µm

20



21

ever, Karmanova (1968) reported that larvae of *E. excisus* were only 140–184 μm long.

Karmanova (1965) reported that the first molt of *E. excisus* occurred in 27–49 days at approximately 20 C and in 18–20 days at 26–35 C. Although the first molt of *E. tubifex* in oligochaetes was not observed in the present study, second-stage larvae were first seen 30 days PI at 25 C. Compared with Karmanova's findings it is likely that *E. tubifex* undergoes the first molt within 20 days. She also indicated that the second molt of *E. excisus* occurred in 80–82 days (no temperature specified) but did not describe the retention of the cuticles of the first and second molt as seen in the present study. Mace and Anderson (1975) indicated that third-stage larvae of *D. renale* retained the cuticles of the first and second molt in oligochaetes. Third-stage *E. tubifex* from oligochaetes (70–109 days PI) were longer (5.7–13.6 mm) than those of *E. excisus* (5.0–6.2 mm) from oligochaetes (Karmanova, 1968).

Lichtenfels and Stroup (1985), in describing a third-stage *Eustrongylides* sp. larva from a naturally infected tubificid, did not indicate the number of cuticles present on the larva; but the length of the larva (3.89 mm), its morphology, and the degree of development of the reproductive system are consistent with those of an early third-stage larva of *E. tubifex* described herein from experimentally infected oligochaetes. Fagerholm (1982) reported 2 cuticles on a *Eustrongylides* sp. larva (14.5 mm long) from a fish collected in Finland. This was likely a third-stage larva that had retained the cuticle of the second molt.

TABLE V. *Major dimensions of third-stage larvae of* Eustrongylides tubifex *from experimentally infected oligochaetes 70–109 days postinfection incubated at 25 C.*

	Male*	Female*
Number	6	11
Length (mm)	9.6 (5.7–13.9)	10.1 (7.1–11.6)
Width†	117 (104–132)	105 (86–124)
Buccal cavity length	62 (51–70)	65 (45–85)
Nerve ring‡	94 (88–102)	107 (89–125)
Oesophagus length (mm)	3.5 (2.2–4.8)	3.7 (2.5–4.2)
Rectum–intestinal junction to posterior extremity	214 (168–320)	215 (190–250)

* Measurements are mean (range) and are in μm unless indicated otherwise.

† At oesophageal–intestinal junction.

‡ From anterior extremity.

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FIGURES 19–21. Third-stage larvae of *Eustrongylides tubifex* from *Limnodrilus* sp. incubated at 25 C. **19.** Cephalic extremity of male larva 109 days postinfection, lateral view (note: first- [L1] and second-stage [L2] cuticles are retained, visible near mouth). **20.** Caudal extremity of male larva 109 days postinfection, lateral view (note: first- [L1] and second-stage [L2] cuticles are retained, visible near anus). Spicule primordium (sp), ejaculatory duct (ed), rectum (r). **21.** Caudal extremity of female larva 102 days postinfection, lateral view (note: first- [L1] and second-stage [L2] cuticles are also retained). Vulva (v), rectum (r).

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A REVIEW OF THE *PROTOMYOBIA AMERICANA* GROUP (ACARINA: PROSTIGMATA: MYOBIIDAE) WITH DESCRIPTIONS OF *PROTOMYOBIA PANAMENSIS* N. SP. AND *PROTOMYOBIA BLARINAE* N. SP.

F. S. Lukoschus*†, G. J. Jeucken, and J. O. Whitaker, Jr.†
Department of Aquatic Ecology, Catholic University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

ABSTRACT: *Protomyobia panamensis* n. sp. is described from *Cryptotis parva orophila* from Panama. It is the only known *Protomyobia* with ovoid ventromedian setae on the tibia and genua of legs I in males. *Protomyobia americana* McDaniel, 1967, is redescribed from its typical host, *Cryptotis parva,* but mites of the genus *Protomyobia* from *Blarina brevicauda,* formerly also classed as *P. americana,* are here described as *Protomyobia blarinae.* In males of *P. americana, ic 1* is short and thin while *cx I²* is much heavier and longer. In *P. blarinae, ic 1* is longer than *cx I².* These 3 species comprise the *P. americana* host group which are separable in males from all other *Protomyobia* in having setae *sc i* behind level of *sc e,* seta *d 1* short and setiform, *cx I²* heavy with a blunt end, and second claw of legs II without a ventral notch. Derived characters of all known *Protomyobia* species are summarized, and a cladogram of the genus was produced which was similar to the cladogram for the host groups.

Ewing (1938) erected the genus *Protomyobia* based on the species *Myobia claparèdei* Poppe, 1896, from the type host *Sorex vulgaris* (now *araneus*) with the type locality as Central Europe. Ewing's description is based on specimens from *Blarina brevicauda* from near Washington, from *Sorex fumeus* from New Hampshire, and from an unidentified small short-tailed shrew from North Carolina. Jameson (1948) described *Protomyobia claparèdei* (Poppe, 1896) from *Blarina brevicauda* from Ontario and New York, and from *Sorex cinereus* from New York. He mentioned slight differences from the original description of Poppe (1896) and from European specimens borrowed from Radford. He suggested that description of a separate subspecies would be necessary when more data became available.

McDaniel (1967) erected the subspecies *Protomyobia claparèdei americana* based on the position of *sc i* in the male, a character previously mentioned by Jameson. McDaniel stated that *Cryptotis parva* from Texas was the type host but he also included in the subspecies *Protomyobia* from *Blarina brevicauda, Sorex fontinalis, Sorex cinereus, Sorex fumeus,* and *Cryptotis parva.* Fain et al. (1982) elevated *americana* from the subspecies to the species level, but included only specimens from *Cryptotis parva* and *Blarina brevicauda.*

In none of these studies was the ventral side of the male described, and the characters of the mites from the 2 American Blarini genera, *Blarina* and *Cryptotis,* had not been compared. Previous studies indicated that one parasite species was present on a number of different hosts, which in turn led to statements concerning transmission between hosts (Jameson, 1948; Jameson and Dusbabek, 1971; Fain et al., 1982).

We have now examined characters of *Protomyobia* from a number of host species. Our analysis indicates more host specificity than formerly recognized and that specimens from *Blarina brevicauda* and *Cryptotis parva* from North America, both of which had been known as *P. americana,* were different. In addition, we found that *Protomyobia* from *Cryptotis parva orophila* from Panama constituted a new species, although comparison with related species proved to be difficult because previous descriptions lacked detail.

The purpose of this paper is to describe the *Protomyobia* from *Cryptotis parva orophila,* to divide *P. americana* into separate species, and to present the results of our study of the cladistics of *Protomyobia.*

MATERIALS AND METHODS

We examined *Protomyobia* from a number of hosts. Mites from *Cryptotis parva orophila* from Panama were

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* Deceased.
† Department of Life Sciences, Indiana State University, Terre Haute, Indiana 47809.

described as new, and specimens from *Cryptotis parva* and *Blarina brevicauda* from North America were divided into separate species. *Protomyobia americana* was retained for those from *C. parva* although it was redescribed, whereas those from *Blarina brevicauda* were described as new.

The outgroup comparison method of Hennig (1982) was used. The characters in the data matrix (Table V) are arranged in increasingly primitive order, i.e., they occur in more primitive myobiids, but less often in *Protomyobia*. The species are arranged by increasing order of derived characters. A cladogram was prepared for *Protomyobia* using the outgroup method and was compared to a cladogram for the host shrews.

All measurements are in microns. Comparative data for the 3 species in the *americana* group of *Protomyobia* are given in Tables I and II for males and females, respectively.

Types were deposited in the following institutions designated in the text as indicated: Naturhistorisches Museum Wien, Austria (NHM); Indiana State University Collection, Terre Haute, Indiana, U.S.A. (ISU); Rijksmuseum van Natuurlijke Historie, Leiden, The Netherlands (RNH); U.S. National Museum of Natural History, Washington, D.C., U.S.A. (USNM); University of Michigan Museum of Zoology, Ann Arbor, Michigan, U.S.A. (UM); Institut royal des Sciences naturelles de Belgique, Bruxelles, Belgium (ISNB); Zoologisches Museum, Hamburg, Germany (ZMH); Field Museum of Natural History, Chicago, Illinois, U.S.A. (FM); Ohio State University, Columbus, Ohio, U.S.A. (OSU).

RESULTS

A revised diagnosis of the genus is presented, the 2 new species of *Protomyobia* are described below, and *Protomyobia americana* is redescribed.

DIAGNOSIS OF THE GENUS *PROTOMYOBIA*

Protomyobia Ewing, 1938

Broad-bodied mites with width–length relation about 1:1.4. Cuticle of idiosoma finely striated transversely except for genital regions, subcapitulum parts of coxal fields I, and epimera. Legs I with 5 free segments and distinct sessile claws. Tibia I with typical specialized ovoid-shaped medioventral seta in both sexes. Genu with posterolateral ventrally bowed broad apophysis of setal origin. Female with ventromedian seta of genu specialized ovoid-shaped or setiform. Femur posterolaterally with typical specialized seta with platelet-like proximal and finger-like apical part. Trochanter I without hooks or spurs. Legs II–IV with 5 free unspecialized segments. Claw formula I–IV: 2-2-1-1. Posterior claw of leg II smaller than anterior claw with or without ventral, subterminal notch. Chaetotaxy of legs: tarsi 8-7-6-6, tibiae 6-6-6-6, genua 8-7-6-6, femora 6-5-3-3, trochanters 3-2-3-3, coxal fields 3-4-1-1. Dorsal trochanter setae III and IV always short. Dorsal idiosomal setation complete: *e I, v i, v e, sc i, sc e*, laterals 1–5, dorsals 1–5, 3 pairs of genital setae. Supernumerary setae may be pres[illegible] hand setae *l 5*. Shape of se[illegible] with ventral inflation of median part. Penis directed backwards, stout, short and sinu[illegible] and slender

Male genital setae reduced to short setiforms or to pointlike setae, or forming long, external penis guide. Dorsals 1–3 in males shifted to position anterior of genital opening. Shape of *d 1–d 3* distinctly setiform, or *d 1* setiform and *d 2, d 3* pointlike, or *d 1–d 3* pointlike. In female *v i* always short setiform, *sc i* short setiform or subequal to *sc e*. Gnathosoma in both sexes cone-shaped without prominent hooks or spurs. Rostral posterior setae elongate (about twice the length of gnathosoma). Parasites of shrews of subfamily Soricinae.

Genotype: Myobia claparèdei Poppe, 1896.

Protomyobia panamensis n. sp.

With the characteristics of the genus *Protomyobia* Ewing, 1938, *sensu* Jameson, 1948. Largest specimen, a female 390 long. Cuticle of idiosoma dorsally and ventrally finely striated, with exception of genitoanal region, and of slightly stronger sclerotized subcapitulum on dorsal and ventral side. Epimera I fused to subcapitulum, forming a short sternum. Position of *ic* setae indicate that they must be regarded as "innermost coxal setae," not as "intercoxal setae." Ventral scutes and spurs on posterior borders of coxal fields I absent. Epimera partly beneath ventral surface, partly fused to surface without striation.

Male (holotype)

Length including gnathosoma 350, in 10 paratypes 350 (352–380), width 290, in paratypes 238 (215–290).

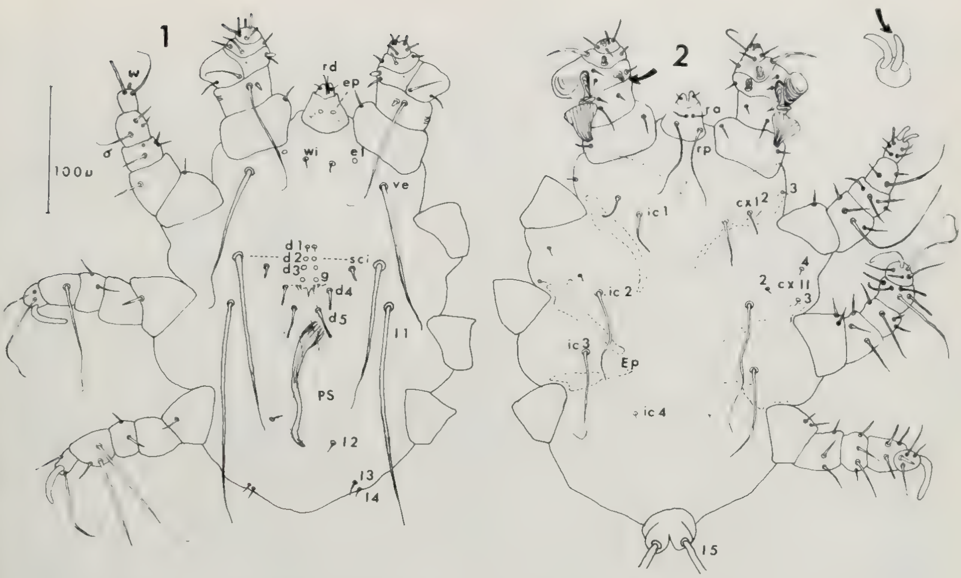
Dorsum (Fig. 1): Present are *e I* (these supracoxal setae of legs I often have been regarded as gland openings); *v i, v e, sc i, sc e, d 1–d 5, l 1–l 5,* and 3 pairs of genital setae. Only *v e, sc e, l 1, and l 5* are formed as long, simple setiforms. Setae *v i* in front of level of *v e*; *sc i* distinctly caudad to level of *sc e*; *d 4* and genital opening craniad of level of *l 1*. Distance from *sc i* to *d 4* (22) only slightly greater than distance from *d 4* to *d 5* (18). File of dorsals shifted between and in front of scapular setae by translocation of genital region. Setae *d 1* in shape of distinct, thin, short setae, *d 2* and *d 3* in shape of pointlike setae, *d 4* and *d 5* (15, 25) are strong setae with blunt ends. Genital region with 2 pairs of thin, short setae and 1 pair of hair rings (g). Setae *l 2–l 4* caudally on opisthosoma (8–10). Aedeagus (105) heavy walled with curved end, directed backwards. A relatively wide penis sheath (PS) connects the penis from its posterior end to the genital opening.

Venter (Fig. 2): Epimera (EP) long, forming almost closed coxal fields, which also include innermost coxal setae. Setae *ic 1–ic 3* long, setiform (35, 80, 95); *ic 4* and coxal setae with exception of *cx* I^2 short setiform (4–8). Second coxal seta (19) with blunt end (appears broken in all specimens at same distance).

Gnathosoma typical for genus with conical stylophore, without gnathosomal hooks. Palps 2-segmented, each segment with 1 seta. Supracoxal setae of palps present (ep), dorsally strong *rd* inserting inside mouth opening, and ventrally *ra* and *rp* very long (60).

Legs evenly spaced laterally with segmentation and chaetotaxy of leg segments similar to related species.

Legs I (Figs. 5, 6) typically shaped for genus with 5 distinctly marked segments and strong, short, sessile claws. Tarsus with *omega* 1 and 8 setae, 5 of which have rounded ends. Tibia ventromedially with ovoid-shaped seta, typical for genera *Nectogalobia* and *Protomyobia*. Genu with posterolaterally and ventrally



FIGURES 1, 2. *Protomyobia panamensis* n. sp., male (holotype). **1.** Dorsum. **2.** Venter.

directed clasping apophysis, 8 setae, and solenidion *sigma.* Ventromedian seta ovoid-shaped as in female. Femur with large modified seta, the apical finger-like part serving together with genu apophysis for clasping host hair.

Legs II–IV subequal, with 5 free segments, pretarsus and larger claws; 2 unequal claws on II, 1 larger claw on legs III and IV. Posterior claw without ventral notch. Legs moderately heavy (length = 5× width of genu). Measurements of paratypes and comparison to related species in Table I.

Chaetotaxy of legs I–IV: tarsi 8-7-6-6, tibiae 6-6-6-6, genua 8-7-6-6, femora 6-5-3-3, trochanters 3-2-3-3. Solenidiotaxy: tarsi 1-1-0-0, genua 1-1-0-0.

Female (allotype)

Length including gnathosoma 375, mean 377 (365–390, n = 10).

Dorsum (Fig. 3): Setae *e I, v i, v e, sc i, sc e, d 1–d 5, l 1–l 5* present; except for *e I* all are simple; filiform without lateral expansions or barbs. Setae *v i* 13, 8 craniad to level of *v e,* which is 120 long; *sc i* (20)

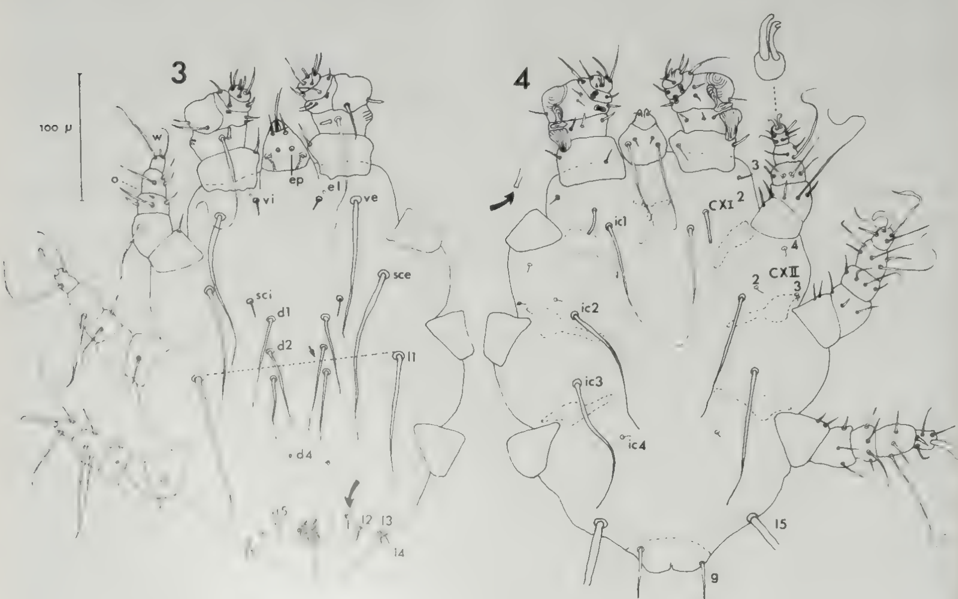
TABLE I. *Measurements of* Protomyobia *males.*

	P. panamensis			*P. blarinae*			
	Type	$\bar{x}$ 10 paratypes	Min–max	Type	3 paratypes	*P. americana* neotype	
Length	350	350	(325–380)	325	325, 336, 350	300	310
Width	215	228	(215–290)	200	210, 218, 225	190	195
v e	115	124	(113–138)	105	105, 110, 115	100	95
sc e	118	122	(105–130)	110	100, 115, 128	110	100
l 1	180	174	(163–180)	165	138, 152, 168	150	155
sc i	11	12	(10–13)	8	8, 9, 10	9	9
d 4	15	16	(13–18)	13	13, 14, 15	14	12
d 5	25	25	(23–28)	24	18, 20, 23	35, 22	30
sc i–d 4	22	24	(22–27)	37	35, 42, 45	38	38
d 4–d 5	18	17	(15–18)	11	10, 12, 13	21	15
l 2	8	9	(8–10)	10	10, 12, 13	11	16
l 3	10	11	(10–13)	14	10, 13, 13	12	12
l 4	[illegible]	9	(8–10)	10	10, 12, 13	11	
ic 1	35	41	(30–50)	50	40, 47, 50	10	10
ic 2	80	87	(70–93)	70	70, 78, 85	65	35
ic 3	95	93	(85–100)	75	70, 79, 90	75	75
ic 4	5	5	(4–5)	5	5, 6, 7	8	[illegible]
cx I	19	20	(17–25)	21	19, 21, 23	125	100

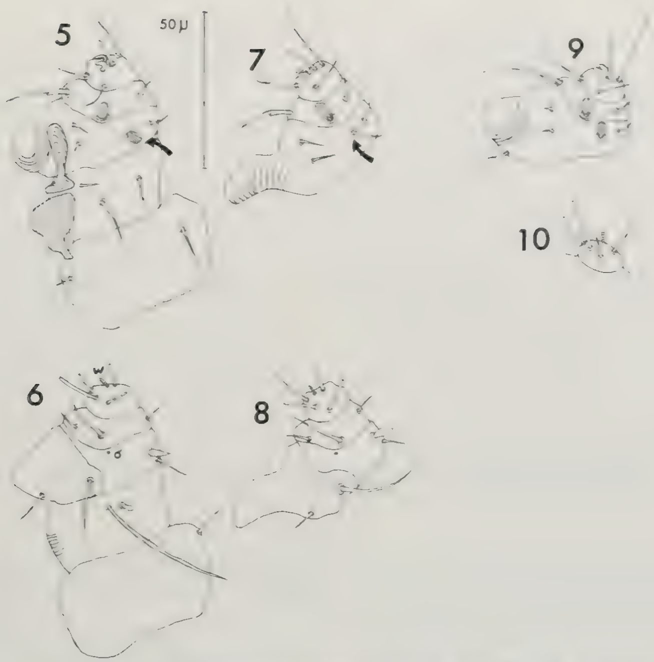
TABLE II. *Measurements of* Protomyobia *females.*

	P. panamensis			*P. blarinae*			*P. americana*		
	Type	$\bar{x}$ 10 paratypes	Min–max	Type	$\bar{x}$ 7 paratypes	Min–max	Neotype	$\bar{x}$ 4 paratypes	Min–max
Length	375	377	(365–390)	445	424	(380–465)	390	386	(355–395)
Width	260	268	(250–275)	300	300	(265–335)	260	228	(210–260)
v i in front of level *v e–v e*	8	8	(7–10)	0	2	(0–8)	3	4	(4–5)
sc i behind level *sc e–sc e*	20	20	(15–28)	30	33	(28–35)	26	30	(28–33)
d 1 in front of level *l 1–l 1*	38	33	(30–38)	17	16	(13–20)	18	22	(17–24)
d 2 in front of (+) or behind (−) *l 1–l 1*	8	+5	(0–+10)	−17	−15	(−13–20)	−12	−14	(−10–15)
d 5 in front of level *l 2–l 2*	8	10	(8–13)	30	31	(28–35)	26	31	(26–35)
d 1	67	63	(55–78)	58	56	(45–67)	65	60	(55–65)
d 2	62	57	(48–65)	55	54	(50–60)	58	50	(48–58)
d 3	50	48	(43–57)	45	44	(40–55)	40	35	(30–40)
d 4	8	10	(5–13)	14	12	(8–15)	16	13	(12–16)
d 5	17	17	(15–23)	28	29	(28–32)	22	24	(21–26)
ic 1	82	91	(70–105)	110	118	(100–130)	80	80	(70–85)
ic 2		101	(85–125)	110	118	(100–135)	100	100	(70–100)
ic 3	105	103	(95–113)	120	116	(100–135)	95	95	(80–120)
ic 4	6	7	(6–7)	8	8	(6–9)	6	6	6
Distance									
d 1–d 1	40	39	(35–44)	60	58	(50–60)	44	43	(42–45)
d 2–d 2	40	38	(36–40)	52	50	(46–54)	48	47	(45–49)
d 3–d 3	40	39	(38–41)	50	46	(43–50)	48	47	(44–48)
d 4–d 4	30	31	(26–34)	37	35	(29–40)	21	24	(20–28)
l 1–file *d*	59	58	(54–62)	53	51	(46–55)	50	43	(38–53)

caudad to level of *sc e*, which is 140 long; dorsals 1–3 (67, 62, 50) distinctly longer than distance *d 1–d 3*. Setae *l 1* 140 long and 38 behind level of *d 2*; *d 4* short (8). Setae *d 5* relatively thin and short for genus, positioned far caudally, laterad of genital region; it is distinctly in front of genital complex in related species. Distance *d 1–d 1* subequal to distance *d 3–d 3*. Distance between dorsal setae distinctly less than distance from lateral 1 to the file of dorsal setae (in allotype 40:59, in 10 paratypes 39:58). Laterals 2–4 short, arranged in



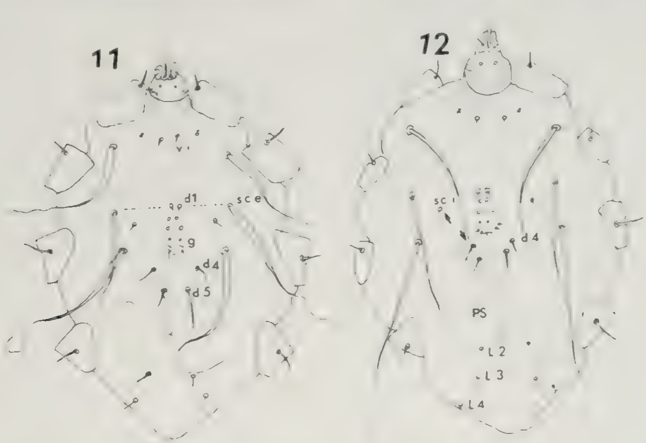
FIGURES 3, 4. *Protomyobia panamensis* n. sp., female (allotype). **3.** Dorsum. **4.** Venter.



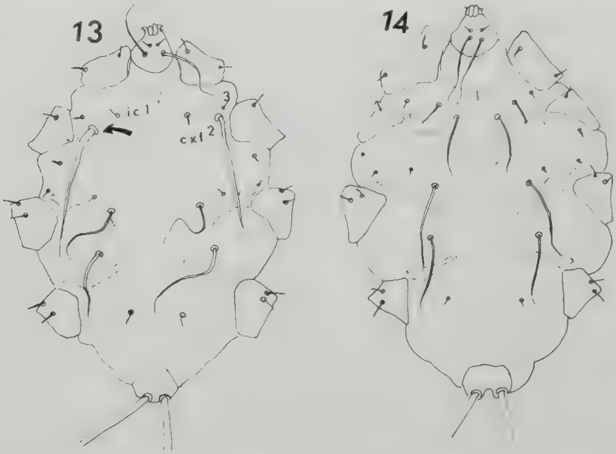
FIGURES 5–10. Leg I of *Protomyobia panamensis* male in ventral view (Fig. 5), and in dorsal view (Fig. 6); tarsus-genu of leg I of *Protomyobia blarinae* male in ventral view (Fig. 7), and in dorsal view (Fig. 8). *Protomyobia panamensis* female leg I tarsus-genu in ventral view (Fig. 9), and in dorsal view (Fig. 10).

an almost transverse rank. Setae *d 5* only 8 in front of level of *l 2*. Genital region similar to that of related species with large vulvar valves, unmodified anal and genital setae, without visible chitinized bursa copulatrix, but with internal sclerotizations. Only 1 pair of genital setae (g) present on unstriated genital cone.

Venter (Fig. 4): Similar to male, but epimera shorter, *ic 1* subequal to *ic 2* and *ic 3*. Ventral opisthosomal opening or tube absent. Gnathosoma and legs similar to that of male, but setae slightly longer. Posterior claw of leg II with distinct notch. Legs I (Figs. 9, 10) with an ovoid seta both on tibia and genu, but with only 3



FIGURES 11, 12. Dorsum of male of *Protomyobia americana* McDaniel, 1967, neotype (Fig. 11) and of *P. blarinae* n. sp. holotype (Fig. 12).



FIGURES 13, 14. Venter of male of *Protomyobia americana* McDaniel, 1967, neotype (Fig. 13), and of *P. blarinae* n. sp. holotype (Fig. 14).

round-pointed setae on tarsus. Measurements of paratypes and comparisons in Table II.

Deposition of types: Holotype male, allotype female and paratypes in USNM, paratypes in NHM, ISU, RHN, USNM, UM, ISNB, ZMH, FM, and OSU.

Material examined: Ex Cryptotis parva orophila Allen, 1895, Cerro Punta, 2,700 m sea level, Prov. Chiriquí, Panama, Graetz legit. Host in collection of NHM Wien, no. 14554, 28 ♂, 31 ♀, 3 larvae, 65 nymphs.

Etymology: The species name refers to the host locality.

Protomyobia americana McDaniel

Protomyobia claparèdei americana McDaniel, 1967: 602.

McDaniel did not give figures or measurements and the type specimen was not deposited in the U.S. National Museum of Natural History. Paratypes or type specimens from the typical host species are not present in National Museum Collection, nor does Dr. McDaniel have them. We thus are redescribing the species and will designate a neotype.

With the characters of the genus *Protomyobia* Ewing, 1938; closely related to *P. panamensis.* Small species, the largest specimen, a female 395 long.

Male (neotype)

Length including gnathosoma 300, in a paratype 310, width 190 (195).

Dorsum (Fig. 11): Setae *v e* (100), *sc e* (110), and *l 1* (150) broader in basal part (5–6) than in previous species. Setae *v i* 12 separated on level of short, 2-knobbed *e 1*. Genital opening at level of *l 1* with 2 pairs of thin setiform genital setae laterad to opening and 1 pair of pointlike setae in front of opening. Dorsals 1 distinctly setiform at level of *sc e*. *d 2* and *d 3* pointlike. *d 4* much closer [illegible] The 2 *d 5* in neotype of different length [illegible] in paratype 30, 30. Laterals 2–4 thin (11–12). [illegible] back-wards with typical shape for the genus: thick-walled, sinuous with curved end. Penis sheath in neotype indistinct.

Venter (Fig. 13): Cuticle and epimera similar to that described for genus and as in *P. panamensis.* Setae of coxal field I of unusual length relation: *ic 1* short (10), second coxal seta (*cx* I^2) heavy and 125 long. This is a secondary modification of *cx* I^2 much longer and stronger than *cx* I^1 (*ic 1*): this condition is not known from any other myobiid species. Setae *ic 2* (65), *ic 3* (75), and *ic 4* (8). Short setae in coxal fields I and II of normal length and relation as in female and in other described species. Terminal setae *l 5* (340) longer than body. Gnathosoma with 55 long *rp* setae. Legs I, shape of ventromedian seta of genu setiform (Fig. 7, arrow). Setation of legs and shape of leg setae and of claws similar to *P. panamensis.*

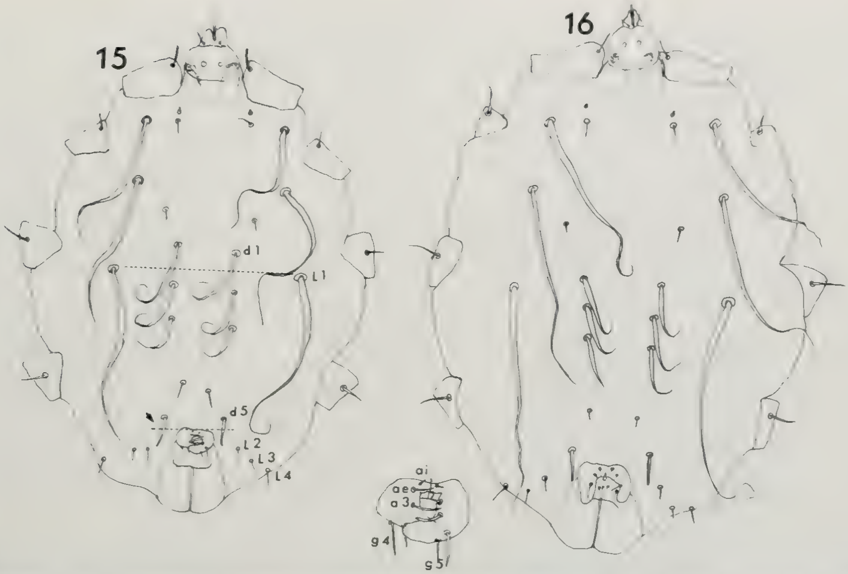
Female

Idiosoma 390 long, 260 wide. General shape broad (width/length 1:1.5).

Dorsum (Fig. 15): Setae *v e* (105), *sc e* (120), and *l 1* (160) strong, setiform. Setae *v i* slightly ahead (2) of level of *v e*; *sc i* distinctly behind (26) level of *sc e*; level of *l 1* between *d 1* and *d 2*. Dorsals 1–3 (65, 68, 40) broadened in median part, distance *d 1*–*d 1* (44) somewhat less than distances *d 2*–*d 2* and *d 3*–*d 3* (48, 48). Distance between dorsals slightly less than distance to *l 1*. Setae *d 4* short (16), *d 5* (22) distinctly in front of genital region. Lateral setae 2–4 in transverse rank. Genital region with soft vulvar valves and the usual setae.

Venter (Fig. 17): Venter, gnathosoma and legs as defined above for genus. Characteristics of venter almost identical to *P. panamensis.* Measurements in Table II indicate comparisons to related species. Gnathosoma and legs lacking outstanding differences.

Deposition of types: Neotype in USNM, paratypes in RNH, ISU, and UM.



FIGURES 15, 16. Dorsum of female of *Protomyobia americana* McDaniel, 1967 (Fig. 15), and of *P. blarinae* n. sp. allotype (Fig. 16).

Material examined: From *Cryptotis parva* Say, 1823, Terre Haute, Vigo Co., Indiana, 10 November 1969, Whitaker, JOW 5426, 2 ♀; ♀ same host, 2 mi E College Station, Brazos Co., Texas, 18 June 1970, neotype male, JOW 5890, collected by J. O. Whitaker, Jr. From same host in Smithsonian collection, no. 197 050, from North Rose, Wayne Co., New York, A. C. Weed coll., 1 ♂, 2 ♀, 3 nymphs, 2 larvae.

Protomyobia blarinae n. sp.

Protomyobia claparèdei (Pope, 1896), Ewing, 1938: 184; Jameson, 1948: 336.

Protomyobia blarinae is closely related to *P. panamensis* and is one of the larger species of the genus; largest specimen, a female 465 long.

Male (holotype)

Length including gnathosoma 325, width 200. Three paratypes measure 325, 337, 350 long and 210, 217, 225 wide.

Dorsum (Fig. 12): Setae *v e* (105), *sc e* (110), and *l 1* (165) smaller in basal part (4) than in *P. americana*; *v i* separated (21) between levels of *v e* and supracoxals *e l*; *sc i* (8) distinctly behind level of *sc e*; genital opening on level of *l 1*. Two pairs of thin setiform genital setae laterad, 1 pair of pointlike genitals in front of genital opening behind file of dorsals 1–3. Setae *d 1* at level of *sc e* distinctly setiform, *d 2* and *d 3* acute. Dorsal 4 (14) and *d 5* (24) situated close together (11), well separated from *sc i* (37). Length of setae *l 2*–*l 4* (10, 14, 10), positioned in diagonal lines from paradian region at level of trochanters IV to sides of opisthosoma. Penis (100) of typical shape for genus with distinct penis sheath (PS) directed backwards to genital opening, thus penis outside of sheath.

Venter (Fig. 14): Cuticle, epimera, and general shape as in *P. panamensis*. Setation of coxal field with *ic 1* 50 long and *cx* I^2 about 20 with blunt end similar to *P. panamensis*, but quite different from *P. americana*. Setae *ic 2* (70), *ic 3* (75), *ic 4* (5), *l 5* (325). Legs I with ventromedian seta of genu setiform (Fig. 7) (ovoid-shaped in male of *P. panamensis*; Fig. 5). Gnathosoma broad, almost rectangular. Legs I with ventromedian seta of genus setiform. Posterior claw of legs II with ventral subterminal notch. Chaetotaxy of legs as described for genus.

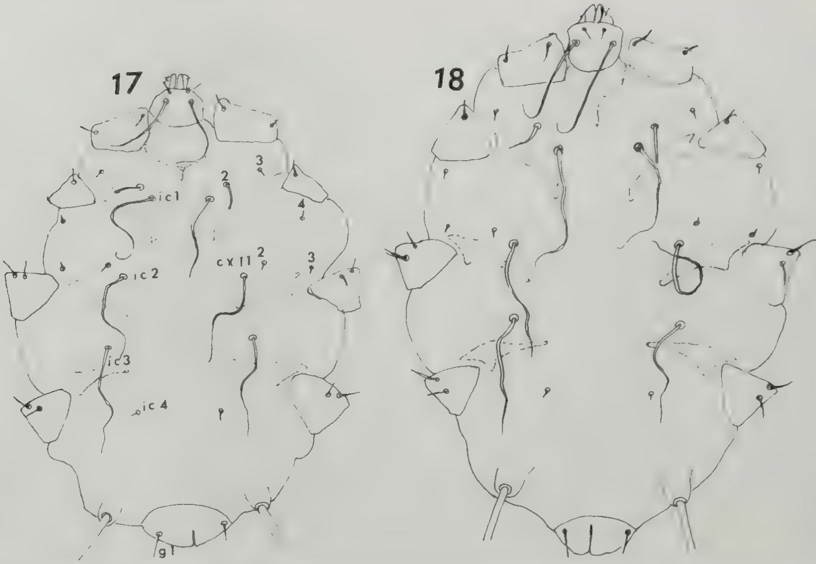
Female

Females averaging 445 long and 300 wide (measurements in Table II).

Dorsum (Fig. 16): Setae *v e* (125), *sc e* (155), and *l 1* (170) with basal part somewhat smaller (4–5) than in *P. americana* (6); *d 1*–*d 3* in median part slightly broadened (4–5) and of decreasing length (58, 55, 45). Level of *l 1* midway between *d 1* and *d 2*. Distance *d 1*–*d 1* greater than distance *d 3*–*d 3*, and greater than distance of dorsal file to *l 1* (53). Setae *d 4* thin (14), *d 5* heavier, with blunt end (29), *l 2*–*l 4* (12–16) in transverse rank. Genital region dorsoterminally with the usual setae *a i, a e, a 3, g 1, g 4, g 5*.

Venter (Fig. 18): Similar to *P. panamensis* and *P. americana*. Measurements *ic 1* (110), *ic 2* (110), *ic 3* (120), *ic 4* (8), *cx* I^2 (28), and relatively heavy with blunt end, *l 5* (360). Gnathosoma, legs I, chaetotaxy, and shape of claws as in *P. panamensis*.

Deposition of types: Holotype and allotype in USNM.



FIGURES 17, 18. Venter of female of *Protomyobia americana* McDaniel, 1967 (Fig. 17), and of *P. blarinae* n. sp. allotype (Fig. 18).

paratypes in ISU, RNH, UM, ISNB, ZMH, FM, and OSU.

Material examined: Ex Blarina brevicauda Say, 1823. Winnipeg, Canada, 7-IX-1969, van Bronswijk leg., holotype ♂, 1 ♂, 1 ♀, 11 immatures; Mt. Carleton Park, New Brunswick, 16 June–24 July 1980, B. M. OConnor leg. Allotype ♀, 1 ♀; Welland Co., Ontario, 24 August 1946, Jameson leg., 1 ♀; Terre Haute, Indiana, 17-IX-1973, E. J. Spicka leg., 1 ♂, 1 ♀; De Bruce, Sullivan Co., New York, 30 September 1976, R. Stehn leg., 3 ♀, 1 ♂; Freeville, New York, 6 November 1970, OConnor leg., 1 nymph; U.S.A. no dates, MNHN Paris, 1 ♀.

COMPARISON TO RELATED SPECIES

Males of the 3 species mentioned above, all from hosts of the tribe Blarinini, share the following characters that separate them from all other *Protomyobia* species parasitic on hosts of tribe Soricini:

1) setae *sc i* distinctly behind level of *sc e*,
2) seta *d 1* distinctly short, setiform,
3) *cx I^2* relatively heavy with blunt end,
4) second claw of legs II without ventral notch.

The 3 known species from hosts of the Neomyini differ from those from Blarinini and Soricini by:

1) second claws of legs II without ventral notch in both sexes,
2) *sc i* in female long, similar to *d 1* in length and shape,

TABLE III. *Diagnostic differences among species of* Protomyobia.

	P. panamensis	*P. blarinae*	*P. americana*
Males			
[illegible]	Ovoid	Setiform	Setiform
[illegible]	Long	Long	Short
[illegible]	Short	Short	Long, strong
[illegible]	ca. 1:1.5	ca. 1:3.5	ca. 1:2.5
Females			
Length	[illegible]	424	380
Level of [illegible]	[illegible]	Between *d 1* and *d 2*	Between *d 1* and *d 2*
Relation distance [illegible]			
[illegible]	[illegible]	>1	<1
[illegible]	[illegible]	>1	<1

TABLE IV. *Character state polarities in genus* Protomyobia.

Primitive (0)	Derived (1)
1. In female *v i* long and similar to *v e*	*v i* reduced to short, thin setae, quite unlike *v e*
2. Apical leg I segments articulated	Distinct, short segments without mobility
3. In female *sc i* long, similar to *d 1*	*sc i* reduced to short, thin setae
4. In female ventral opisthosomal tube present	Tube absent
5. In female *ic 4* long, similar to *ic 3*	*ic 4* much shorter than *ic 3*
6. In female posterior claw II ending in 1 point	Claw with distinct ventral notch
7. In male setae *d 2, d 3* short setiform	Setae reduced to pointlike setae or hair rings
8. In male *ic 1, ic 2,* and *ic 3* long and subequal	*ic 1* distinctly shorter than *ic 2*
9. In male *d 1* in shape of short, distinct setae	*d 1* reduced to pointlike setae or hair rings
10. In male posterior claw II 1-pointed	With distinct ventral notch
11. In female *d 1–d 3* longer than 18	Length of setae reduced, shorter than 14
12. In female *ic 1* long and subequal to *ic 2*	*ic 1* strongly reduced in length
13. In female ventral seta on genu I ovoid (derived character state of *Nectogalobia–Protomyobia* group)	All ventral genu I setae setiform (secondary reversal)
14. In male ventral seta on genu I setiform	Ventral genu I seta ovoid-shaped
15. Coxal setae setiform with pointed or whiplike end	$cx\ I^2$ seta with blunt end
16. In male *ic 2* subequal to *ic 3*	*ic 2* distinctly shortened
17. In female opisthoventer with none or only 1 pair of supernumerary setae	6–10 pairs of supernumerary setae
18. In male $cx\ I^2$ shorter than *ic 1*	$cx\ I^2$ longer and stronger than *ic 1*
19. In female setae *d 1–d 3* setiform	Flattened in their basal or middle parts
20. Claws III and IV slender	Claws broadened and flattened

3) opisthosomal tube is present in female venter, 4) setae *d 1–d 3* in male setiform.

The 3 species from American Blarinini form a subgroup (*americana* group) with characters intermediate between those from hosts from the Neomyini and Soricini.

There are few differences among females of the species of the *americana* group, but differences are evident in the males: *Protomyobia panamensis* n. sp. is the only known *Protomyobia* species with ovoid-shaped setae ventromedially both on the tibia and genu of legs I in males. The males of all other species have only the tibial seta modified. In *P. americana,* the male has the *ic 1* short and thin, while $cx\ I^2$ is much heavier and longer, whereas the general tendency in coxal field I is for decreasing length from *ic* to the more laterad coxal setae. A similar inversion of length and shape of the coxal setae is present in coxal fields II–IV in the subgenus *Rattimyobia* from Southeast Asian *Rattus* (Fain et al., 1980).

Diagnostic differences are summarized in Table III.

CLADISTIC RELATIONS IN THE GENUS *PROTOMYOBIA*

We have attempted to arrange the species of the genus *Protomyobia* into a cladistic system. *Nectogalobia sinensis* was chosen as an outgroup and is regarded as the most primitive myobiid from insectivoran hosts (Fain and Lukoschus, 1967). This species shares with *Protomyobia* a number of derived characters including those secondarily modified, not present in other gen-

TABLE V. *Data matrix of* Protomyobia *species.*

	Characters																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Nectogalobia sinensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Protomyobia kounickyi	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
P. nodosa	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
P. nepalensis	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
P. blarinae n. sp.	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0
P. americana	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0
P. panamensis n. sp.	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0
P. claparedei	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
P. onoi	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
P. nipponensis	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
P. indianensis	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0
P. brevisetosa	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0

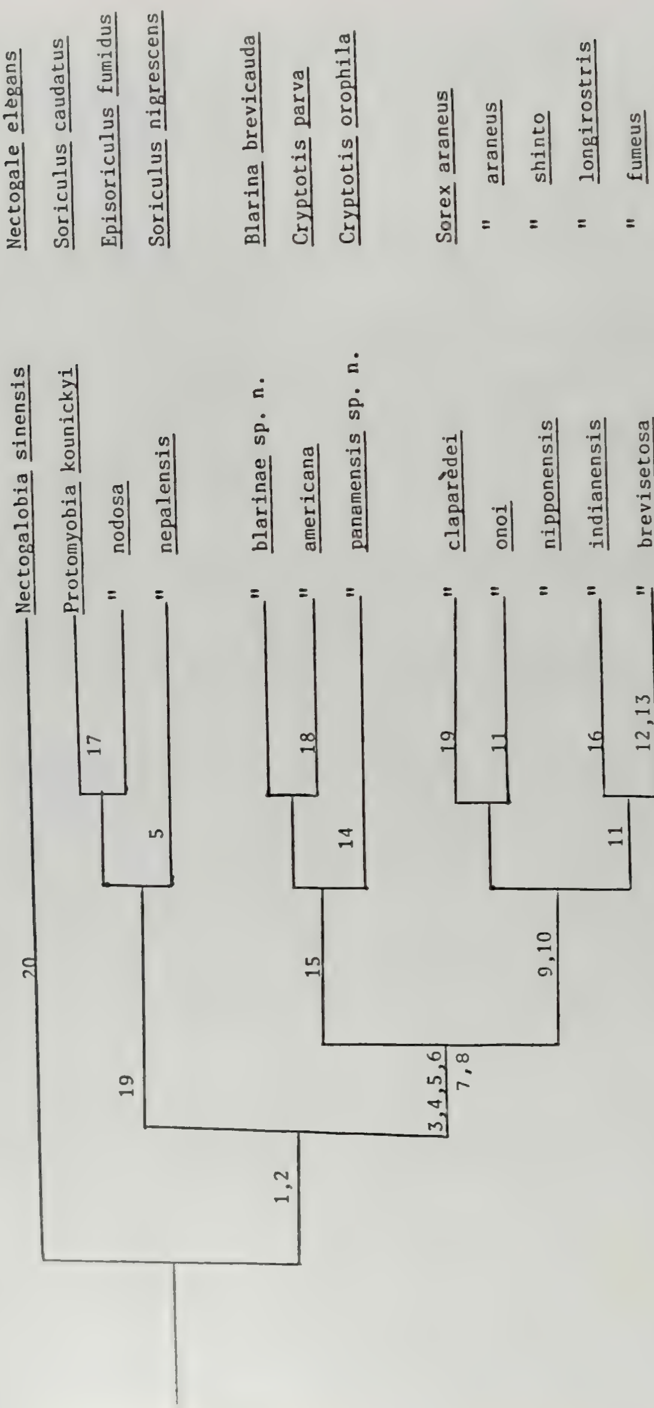


FIGURE 19. Cladistic arrangement of *Protomyobia* species.

era: the ovoid-shaped setae medioventrally on tibia and genu I of the female, and the specialized seta with a finger-like apical end posterolaterally on femur I. *Nectogalobia sinensis* possesses the primitive character states of *Protomyobia* species, as given in our Tables IV and V; characters 3–15, and also some additional primitive characters not present in *Protomyobia* in the derived state (1 and 2). These character state polarities are listed in Table IV. Although helpful, characters of the developmental stages were omitted because they are not available for all species. Arrangement by derived characters, mostly reduction characters, resulted in grouping as shown in the data matrix (Table V).

There is an evident reduction line from the outgroup *Nectogalobia,* which has all idiosomal setae on the dorsum subequal in shape and all innermost coxals long, through *Protomyobia nodosa, kounickyi,* and *nepalensis* with a reduction of *v i,* then through species from the Blarinini hosts, which had *sc i,* the ventral tube, and *ic 4* reduced, to the parasites of the genus *Sorex.* Reductions of the length of the dorsal setae, of *ic 1,* and of genu I seta separate species in the *Sorex* group. In males, dorsals 1–3 are setiform in the species from *Nectogale* and Soricina hosts. In Blarinini hosts only *d 1* is setiform with *d 2* and *d 3* reduced to pointlike setae or hair rings, while all species from *Sorex* have these 3 pairs of dorsals reduced to small setal rings. When we arranged the groups of characters of the data matrix into a cladistic system (Fig. 19), the relation of character combinations to the systematic groupings of hosts became more evident.

In his study of the family Soricidae, Repenning (1967) stated that the tribe Neomyini arose in the late Oligocene and Blarinini arose in the middle part of the Miocene, while speciation of the Soricini occurred in the Pliocene (Hoffman, 1971; George, 1984). These historical dates regarding evolution of the hosts parallel the accumulation of derived characters in the parasites. The parasites appear to have evolved with their hosts. Early offshoots from the Soricinae have parasites with primitive characters apparently not much modified since divergence, whereas later offshoots show larger numbers of derived characters.

DISCUSSION

The cladistic arrangement of *Protomyobia* species appears to contradict ideas on distribution of American *Protomyobia* species as suggested by Jameson and Dusbabek (1971) and Fain et al. (1982). These authors suggested that the Eurasian species *P. claparèdei* and, later, *P. onoi* entered North America as parasites of the holarctic species *Sorex arcticus* and *S. cinereus* and more recently moved onto *Blarina* and *Cryptotis.* If so, these mite species should have adapted to *Blarina brevicauda* after migrating from *Sorex cinereus.*

In view of our information on the morphology of insectivore myobiid species, this theory becomes questionable. The myobiids from Blarinini (*Blarina* and *Cryptotis*) have more complete setation than those from Soricini and thus would appear to represent the more primitive form. A species showing evolutionary reductions on a host group undergoing recent speciation (*P. claparèdei–onoi* group from *Sorex cinereus*) cannot be expected to redevelop lost characters even in adapting to an older branch of the host family. This would be a case of reversibility of evolution, improbable according to the rule of Dollo (1893). On the contrary we would expect coevolution of hosts and their parasites in host-specific parasites (Eichler, 1941). *Sorex* evolved more recently with an evolutionary center in the Bering Strait region (Hoffmann and Peterson, 1967); thus precursors of *Sorex* probably were parasitized by precursors of the present *Sorex* parasite species. Speciation of parasites is generally slower than speciation of hosts. In host groups undergoing recent speciation, parasites should show smaller morphological differences, while in host groups that originated earlier, parasites should show greater differences, assuming equal rates of evolution (Fahrenholz, 1916; Szidat, 1956; Fain, 1975). In closely related host groups, forms from different hosts will generally be very similar and would be regarded as conspecific or perhaps distinct only subspecifically (Fain and Lukoschus, 1977).

Besides the species of *Protomyobia,* members of the family Soricidae are parasitized by a second myobiid genus, *Amorphacarus.* This genus appears to be more mutagenic, showing greater reductions. A parallel comparative study of the evolution in *Amorphacarus* from many of the same hosts is in preparation.

Based on this study we now propose the division of the genus into 3 subgenera as follows:

1) *Protomyobia* Ewing, 1938

As in the definition of the genus. In addition in the female *sc i* short, ventral opisthosomal tube absent, *ic 4* short. In male *ic 1* shorter than

ic 2, penis short, stout and sinuate; genital setae minute; *d 1–d 3* pointlike. In both sexes second claw of legs II with subterminal notch. Parasites of shrews of genera *Sorex* and *Blarinella*.

Typical species: Myobia claparèdei Poppe, 1896.

2) *Asioprotomyobia* n. subgen.

As described for the genus. In female *sc i* long, almost subequal to *sc e*; ventral opisthosomal tube present; *ic 4* subequal to *ic 3* or shorter. In male *d 1–d 3* distinct setiform; *ic 1, ic 2, ic 3* long and subequal; penis long and slender or short and stout; genital setae forming external penis guide or short. Second claw of legs II in both sexes without notch. Parasites of Neomyini.

Typical species: Protomyobia nepalensis Fain and Lukoschus, 1980.

3) *Amprotomyobia* n. subgen.

With the definition of the genus. In female *sc i* short; *ic 4* short; ventral opisthosomal tube absent; posterior claw of leg II with subterminal notch. In males *d 1* setiform, *d 2* and *d 3* pointlike; penis short and stout; genital setae short; posterior claw of leg II without notch. Parasites of *Blarina* and *Cryptotis*.

Typical species: Protomyobia claparèdei americana McDaniel, 1967.

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EIMERIA FROM BATS OF THE WORLD. II. A NEW SPECIES IN *TADARIDA FEMOROSACCA* FROM SONORA, MEXICO

Donald W. Duszynski, David W. Reduker*, and Brent B. Parker†
Department of Biology, The University of New Mexico, Albuquerque, New Mexico 87131

ABSTRACT: Between 1979 and 1980, 104 bats representing 13 species in 4 families were collected in California and New Mexico, U.S.A., and Baja California and Sonora, Mexico, and were examined for coccidia; only 3 (3%) had oocysts in their feces. Bats examined and their infection rates were: Molossidae: 0 of 12 *Tadarida brasiliensis*, 1 of 18 (6%) *T. femorosacca*; Natalidae: 0 of 1 *Natalus stramineus*; Phyllostomatidae: 0 of 1 *Choeronycteris mexicana*, 0 of 2 *Leptonycteris sanborni*, 0 of 1 *Macrotus californicus*; Vespertilionidae: 0 of 9 *Antrozous pallidus*, 0 of 28 *Eptesicus fuscus*, 0 of 1 *Lasionycteris noctivagans*, 0 of 3 *Lasiurus borealis*, 2 of 22 (9%) *L. cinereus*, 0 of 1 *L. ega*, 0 of 5 *Pipistrellus hesperus*. Sporulated oocysts were only found in *T. femorosacca* and these represent a new species, *Eimeria tadarida* n. sp. They are subspheroidal to ellipsoidal, 19 × 25 (16–23 × 20–30) μm; a micropyle is absent, and fragments within the oocyst may be oocyst residuum or multiple polar bodies. The oocyst wall, ~1.5 μm, is composed of a mammillated outer layer and smooth inner layer. Sporocysts are ovoidal, 8 × 12 (6–9 × 10–14) μm, and have a small Stieda body and a wide substieda body. This is only the 14th eimerian to be described from bats worldwide. Only unsporulated or partially sporulated oocysts of an eimerian were seen in 2 *L. cinereus*. These measured 28 × 25 (27–29 × 24–26) μm and had a mammillated outer oocyst wall.

From July 1979 through December 1980 bats were collected from various localities in California and New Mexico, U.S.A., and Baja California and Sonora, Mexico; all animals were examined for the presence of coccidian oocysts in their feces. This paper summarizes our findings.

MATERIALS AND METHODS

Hosts were captured alive in mist nets and killed within a few hours after capture. Procedures for removal of intestines, preserving fecal material, and processing, measuring, and photographing oocysts were as described previously (Duszynski et al., 1982; Stout and Duszynski, 1983). Skeletons, skins, and tissues for isoenzyme analysis for all hosts are permanently deposited in the Museum of Southwestern Biology, UNM. All measurements are in μm with means ± 1 SD in parentheses following the ranges.

RESULTS

Of 104 bats representing 13 species and 4 families from 14 collection localities (Table I) only 3 had large numbers of oocysts in their feces. The study of these oocysts revealed qualitative and quantitative differences between them and oocysts of other *Eimeria* spp. described from bats.

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* Present address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164.

† Present address: College of Veterinary Medicine, Colorado State University, Ft. Collins, Colorado 80523.

Eimeria tadarida n. sp.
(Figs. 1–4, 8)

Description

Oocysts subspheroidal (Fig. 1) to ellipsoidal (Fig. 2), wall of uniform thickness ~1.5, with 2 layers: outer layer mammillated (Fig. 3), ~⅔ of total thickness, inner layer smooth; micropyle absent; 1–3 fragments within oocyst (Fig. 4) that may represent multiple polar bodies or a dispersed oocyst residuum; sporulated oocysts (n = 100) 20–30 × 16–23 (25.2 ± 2.1 × 19.0 ± 1.2), with L:W ratio 1.2–1.6 (1.3 ± 0.1); sporocysts (n = 100) ovoidal, 10–14 × 6–9 (12.1 ± 0.7 × 7.6 ± 0.9), with L:W ratio 1.4–1.7 (1.6 ± 0.1); Stieda body small, difficult to see with transmitted light or interference contrast microscopy; substieda body apparently present (there is always a clear space below pointed end of sporocyst: Figs. 1, 2, 4), asymmetrical, 2–3× wider than Stieda body; parastieda body absent; sporocyst residuum composed of several small to large globules and granules, sometimes obscuring sporozoites; sporozoites with a large, posterior refractile body. Oocysts were 1,935 days old when measured.

Taxonomic summary

Diagnosis: Of the 13 valid species of *Eimeria* previously described from chiropteran hosts (Levine and Ivens, 1981; Duszynski and Barkley, 1985), only *Eimeria tomopea* Duszynski and Barkley, 1985, *Eimeria macyi* Wheat, 1975, and *Eimeria eumopos* Marinkelle, 1968 resemble *E. tadarida*. Oocysts of *E. tadarida* differ from those of *E. tomopea* by being smaller and lacking a polar body; they also have smaller sporocysts that have a substieda body that *E. tomopea* lacks, although in both species the Stieda body is difficult to visualize. They differ from those of *E. macyi* by having a residuum and lacking polar bodies; by having larger oocysts with a larger L:W ratio (1.3 vs. 1.1); and by having a substieda body that is 2–3× wider than the Stieda body vs. one that is the same width. They differ

TABLE I. *Thirteen species of bats representing 4 families taken from 14 collection localities from California, New Mexico, and Baja California and Sonora, Mexico and examined for coccidia.*

Chiropteran family/host	Country: County/locality, state	No. hosts infected/ no. examined (%)
Molossidae		
Tadarida brasiliensis	U.S.A.:	
	Socorro Co., New Mexico	0/5
	Hidalgo Co., New Mexico	0/7
T. femorasacca	Mexico:	
	Sierra San Pedro Martir, Baja	0/12
	Mission de San Borja, Baja	0/5
	Alamos, Sonora	1/1
Natalidae		
Natalus stramineus	Mexico:	
	Guaymas, Sonora	0/1
Phyllostomatidae		
Choeronycteris mexicana	Mexico:	
	Rancho Santa Catarina, Baja	0/1
Leptonycteris sanborni	Mexico:	
	San Carlos, Sonora	0/2
Macrotus californicus	Mexico:	
	San Carlos, Sonora	0/1
Vespertilionidae		
Antrozous pallidus	U.S.A.:	
	Madera Co., California	0/6
	Mexico:	
	Punta Chueca, Sonora	0/2
	Sierra San Pedro Martir, Baja	0/1
Eptesicus fuscus	U.S.A.:	
	Hidalgo Co., New Mexico	0/6
	Sandoval Co., New Mexico	0/1
	Socorro Co., New Mexico	0/1
	Mexico:	
	Sierra San Pedro Martir, Baja	0/20
Lasionycteris noctivagans	U.S.A.:	
	El Dorado Co., California	0/1
Lasiurus borealis	U.S.A.:	
	Hidalgo Co., New Mexico	0/3
L. cinereus	U.S.A.:	
	El Dorado Co., California	0/3
	Hidalgo Co., New Mexico	1/8 (12.5)
	Mexico:	
	Sierra San Pedro Martir, Baja	1/11 (9.0)
L. ega	Mexico:	
	Mission de San Borja, Baja	0/1
Pipistrellus hesperus	Mexico:	
	Guaymas, Sonora	0/3
	Sierra San Pedro Martir, Baja	0/1
	Valle de Trinidad, Baja	0/1
4 families, 13 species	14 localities	3/104 (1)

from those of *E. eumopos* by lacking polar bodies and being much smaller (25 × 19 vs. 35 × 28); by having a thinner outer wall that is mammillated, not pitted; and by having sporocysts with a substieda body that *E. eumopos* lacks.

Type host: *Tadarida femorosacca* (Merriam), pocketed free-tail bat, Museum of Southwestern Biology, Division of Mammals, MSB #53835 (female), J. Haydock #282, 27 October 1980.

Type locality: Mexico. Sonora: 19.3 km E Alamos by road Rio Cuckujaqui.

Site of infection: Unknown. Oocysts recovered from feces.

Etymology: The *nomen triviale* is derived from the generic name of the host.

***Eimeria* sp.**
(Figs. 5–7)

Oocysts subspheroidal (Fig. 5), wall of uniform thickness ~2.0, with 2 layers: outer layer mammillated (Fig. 6, insert), ~¾ of total thickness, inner layer smooth (Fig. 6). Although no sporulated oocysts were seen, a few abnormally sporulated forms (Fig. 7) clearly show

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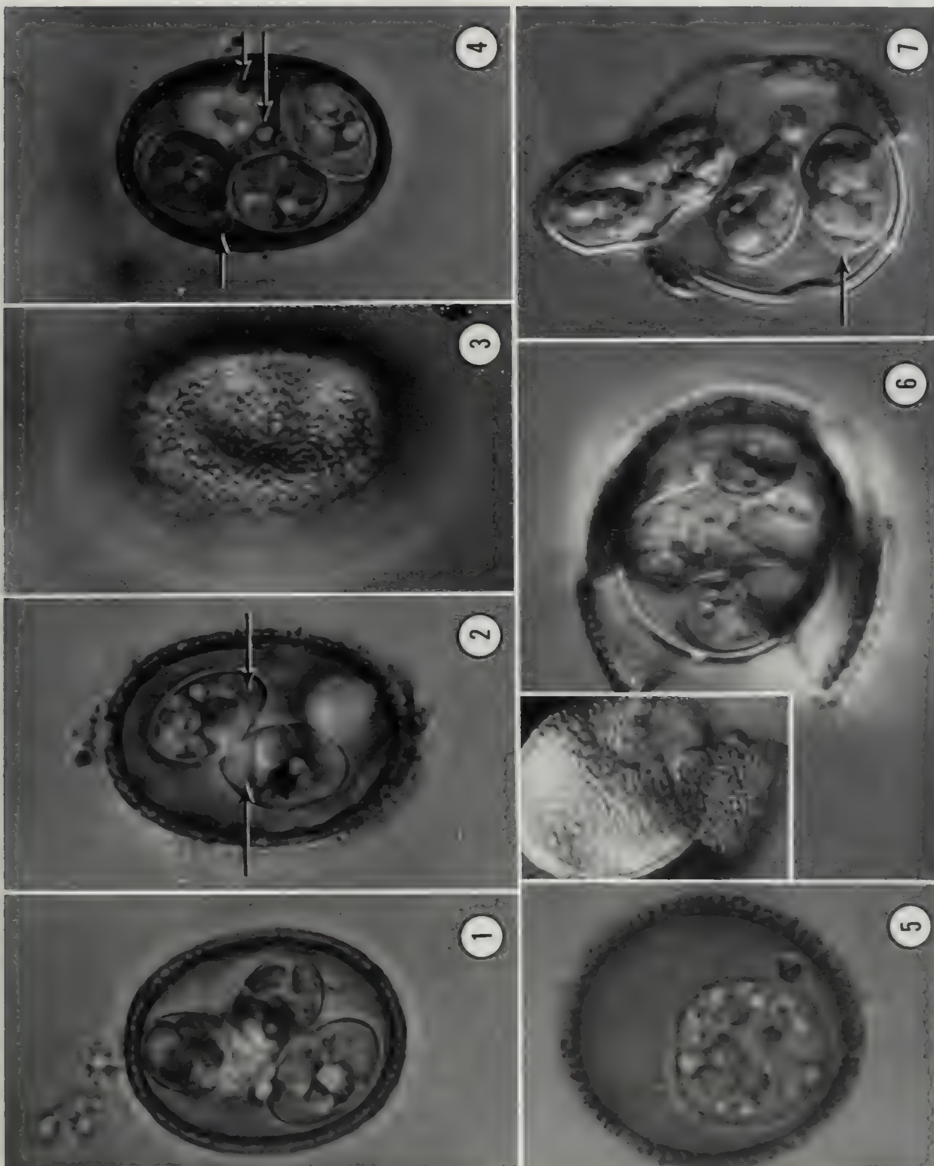
FIGURES 1–7. Photomicrographs of sporulated oocysts of *Eimeria tadarida* n. sp. collected from the feces of *Tadarida femorosacca* (Figs. 1–4) and of unsporulated oocysts of an eimerian collected from the feces of *Lasiurus cinereus* (Figs. 5–7). × 1,600. **1.** Subspheroidal oocyst; note striated appearance of wall. **2.** More ellipsoidal oocyst; note the clear area (arrows) seen in these sporocysts and in those in Figures 1 and 4, that is probably a substieda body. **3.** Mammillated surface of outer oocyst wall (NIC). **4.** Oocyst with what is either multiple polar bodies or fragments of a residuum (arrow). **5.** Subspheroidal oocyst. **6.** Outer wall separating from smooth inner wall of partially sporulated oocyst; insert shows mammillated outer layer of oocyst wall (NIC). **7.** Broken, partially sporulated oocyst with what may be a parastieda body (arrow) in one of the sporocysts.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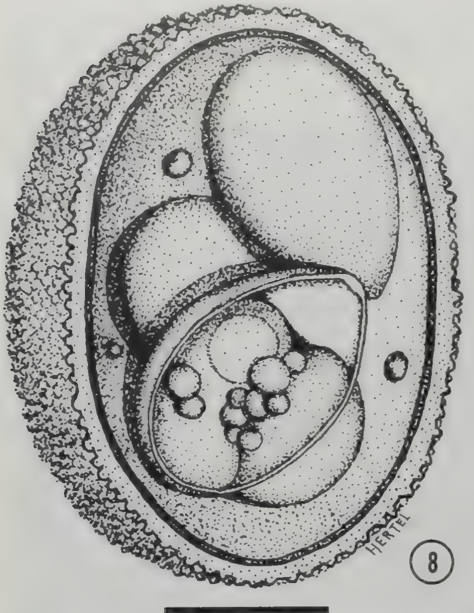

FIGURE 8. Line drawing of a sporulated oocyst of *Eimeria tadarida*. Bar = 5 μm.

that this species is an eimerian. Unsporulated oocysts were 28 × 25 (27–29 × 24–26) with L:W ratio 1.1.

Taxonomic summary

Diagnosis: This form is similar in either size or shape to *E. eumopos*, *E. macyi*, *E. tomopea*, and *Eimeria zakirica* Mussev, 1967. It differs from the first 3 by being smaller than *E. eumopos* (35 × 28) and *E. tomopea* (31 × 25) and larger than *E. macyi* (19 × 18). It differs from *E. eumopos* and *E. tomopea* by being less ellipsoidal (L:W 1.1 vs. 1.2–1.4). It is similar to all 3 species, however, by having a highly sculptured outer wall that appears striated in optical cross section. Although similar in size and shape to *E. zakirica*, the 2 differ because *E. zakirica* has a thin, smooth 1-layered oocyst wall.

Type host: Lasiurus cinereus (Beauvois, 1796), hoary bat, Museum of Southwestern Biology, Division of Mammals, MSB #42509 (male), D. A. Goebel #38, 16 June 1980.

Type locality: U.S.A. New Mexico: Hidalgo Co., 27.3 km S, 10.6 km E, Animas.

Other host and locality: Lasiurus cinereus, MSB #43048 (male), T. L. Best #8622, 8 July 1980, Mexico, Baja California, Parque Nacional, Sierra de San Pedro Martirs.

DISCUSSION

The mammalian order Chiroptera is second only to the rodents in terms of species diversity and numbers. However, in contrast to rodents, few *Eimeria* spp. have been described from bat hosts. For example, only 14 valid *Eimeria* species have been described from 14 species of bats, 1.5% of the living bat host species (see Duszynski and Barkley, 1985, for review). There are at least 2 possible explanations for this. First, as pointed out by Ubelaker et al. (1977), the paucity of *Eimeria* spp. described from bats reflects the fact that few people have examined bats for intestinal coccidia. A second explanation is that bats, as a group, seem not to be regularly parasitized by monoxenous coccidians. In the few surveys that have been done, only small numbers of bats are ever found to be discharging oocysts. For example, Marinkelle (1968) found only 2 of 400 bats (23 species) in Colombia to have oocysts in their feces when examined. Similarly, here we found only 3 of the 104 bats (13 species) we examined from Mexico and the western U.S.A. to be actively discharging oocysts. The mechanism that drives this low rate of infection simply may be the feeding habits of the hosts. Most bats (e.g., molossids, vespertilionids) are insectivores and insects on the wing do not seem to be particularly good concentrating mechanisms for oocysts. Bats that eat other things such as fruit, nectar, or pollen (e.g., phyllostomatids) do not seem to be any more often infected than insectivores, although more survey work needs to be done before this idea can be critically examined. Likewise, *Antrozous*, a bat with unusual eating habits and known to forage on the ground and eat scorpions and crickets, also is not often infected with coccidians. Thus, regardless of diet, bats have never been found to be heavily infected with coccidians. Host genetic factors (e.g., high chromosomal variability) may contribute to this phenomenon (see Duszynski, 1986). Finally, it is interesting to note that 2 *L. cinereus* collected in different localities were discharging oocysts (*Eimeria* sp., above) that probably represent the same species.

Based on characteristics of the oocyst wall (thickness and texture), Duszynski and Barkley (1985) hypothesized at least a diphyletic origin for *Eimeria* spp. infecting bats. The 3 eimerians previously described from New World vespertilionid and molossid bats (*E. macyi, E. tomopea, E. eumopos*) have thick and mammillated oocyst walls, whereas the 6 eimerians described from African and Old World vespertilionids and molossids (*E. dukei, E. levinei, E. nyctali, E. vajsovi, E. vespertilii, E. zakirica*) have smooth-walled oocysts. *Eimeria tadarida* and *Eimeria* sp., which parasitize a New World molossid and

vespertilionid, respectively, both have mammillated oocyst walls, thus supporting this hypothesis.

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TWO NEW SPECIES OF *MONILIFORMIS* (ACANTHOCEPHALA: MONILIFORMIDAE) FROM MALAYSIA

Timothy P. Deveaux, Gerald D. Schmidt, and M. Krishnasamy*
Department of Biological Sciences, University of Northern Colorado, Greeley, Colorado 80639

ABSTRACT: *Moniliformis tarsii* n. sp. was found in *Tarsius bancanus*. It is unique in possessing 11–12 longitudinal rows of 6–7 hooks each. Hooks 2 and 3 are conspicuously enlarged, 41–55 μm long. *Moniliformis echinosorexi* n. sp. differs from all other species in having 12–15 rows of 11–13 hooks that are 34–36 μm long, and in having a proboscis receptacle 1.2–2.0 mm long. Several new host records for *M. moniliformis* are presented.

This report is based on specimens collected by M.K. and associates from the Institute for Medical Research of Malaysia, from 1965 to 1978. Worms were fixed in Bouin's solution or AFA, stained with Semichon's carmine, and mounted in balsam. Drawings were made by use of a microprojector and camera lucida. Measurements are in μm unless otherwise stated. Width measurement indicates greatest width.

Moniliformis tarsii n. sp.
(Figs. 1–8)

Fifteen sexually mature specimens were recovered from 3 Horsfield's tarsiers, *Tarsius bancanus* (Horsfield) (Mammalia: Primates). The tarsiers were collected in Poring National Park, and Kampung Silad, Ranau, Sabah, East Malaysia.

Description

With characteristics of *Moniliformis* as described by Schmidt (1972). Sexual dimorphism of the specimens present but not marked. Pseudosegmentation absent. Armature (Figs. 1–6) similar in both sexes: 11–12 regularly alternating, longitudinal rows of 6–7 hooks. Anterior hook 26–37 ($\bar{x}$ = 31); next 2 hooks 41–55 ($\bar{x}$ = 45); hooks 4–7 24–28 ($\bar{x}$ = 25). First 3 hooks with powerful roots, remaining hooks weakly rooted. Lemnisci flat with 3 giant nuclei each and reach 25–30% of body length. Lemnisci not bound posteriorly to inner body wall.

Male (Fig. 7a, b) (3 specimens): 15–19 mm long, 800–900 wide. Proboscis 490 long, 240 wide. Proboscis receptacle 800 long, 310–350 wide. Testes posterior, in tandem. Anterior testis 1.66–1.88 mm long, 330 wide. Posterior testis 1.5 mm long, 430 wide. Eight cement glands, each with single giant nucleus, located in compact group posterior to testes. Genital pore terminal.

Female (12 specimens): All specimens gravid, 21–41 mm long ($\bar{x}$ = 28), 0.73–1.57 mm wide ($\bar{x}$ = 1.10). Proboscis 490–560 long ($\bar{x}$ = 520), 230–320 wide ($\bar{x}$ = 240). Lemnisci and uterine bell obscured by eggs in all specimens except one; uterine bell 1.03 mm from posterior end. Eggs (Fig. 8) 47–55 long ($\bar{x}$ = 51), 16–21 wide ($\bar{x}$ = 17) as measured through body wall. Outer envelope with some sculpturing.

Type host: Horsfield's tarsier, *Tarsius bancanus* (Horsfield).

Location: Small and large intestines.

Type locality: Poring National Park, Sabah, East Malaysia.

Type specimens: USNM Helm. Coll. holotype male No. 80141, allotype female no. 80142, paratype male No. 80143.

Etymology: Name derived from genus of host.

Taxonomic summary

The relatively large second and third hooks separate these specimens from the majority of *Moniliformis* species. *Moniliformis semoni* (Linstow, 1898) Johnston and Edmonds, 1952, has fourth and fifth hooks that average 68 (Johnston and Edmonds, 1952), but *M. semoni* has 13–15 hooks per row. *Moniliformis spiralis* Subrahmanian, 1927, is approximately the same length as *M. tarsii*, but *M. spiralis* has 12 rows of 10–13 hooks (Petrochenko, 1958; Yamaguti, 1963).

One specimen of *Moniliformis moniliformis* was reported by Brach and Niemitz (1984) from the ileum of a wild-caught tarsier in Ulu Belleh, uplands of the River Belleh, a tributary of the Rajang River, Central Sarawak. This was reported as a new host record, but no description of the specimen was given. Therefore, we cannot tell if the specimen was indeed *M. moniliformis* or *M. tarsii* n. sp.

Moniliformis echinosorexi n. sp.
(Figs. 9–12)

Eight sexually mature specimens were found in 6 moon rats, *Echinosorex gymnurus* (Raffles)(Mammalia: Insectivora) collected in Bukit Legong, Kepong, Selangor (2 specimens); Ampang Forest Reserve, Selangor; Janda Baik, Pahang; and Ulu Gombak, Selangor, Peninsular Malaysia. Male specimens from Washington Zoological Gardens also were sent to our laboratory by Dr. Marion Georgi of Cornell University, whom we thank. The specimens were obtained from a wild-caught moon rat.

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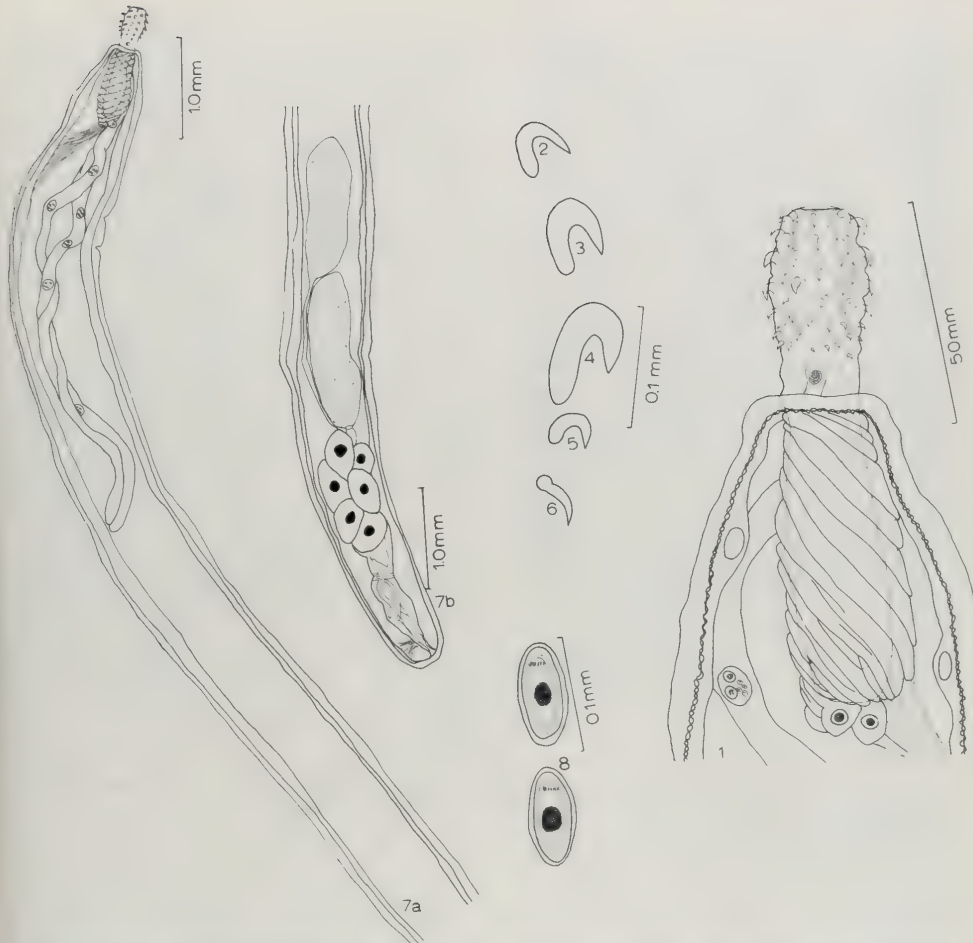
* Division of Medical Ecology, Institute for Medical Research, Jalan Pahang, Kuala Lumpur 50588, Malaysia.



FIGURES 1–8. *Moniliformis tarsii* n. sp. from *Tarsius bancanus.* **1.** Anterior end of male. **2–6.** Lateral view of row of first 5 hooks of same specimen, showing enlarged hooks 2 and 3. (Individual hooks numbered to show location of enlarged hooks.) **7a, b.** Anterior and posterior ends of male body. **8.** Eggs.

Description

All specimens are pseudosegmented. Armature (Fig. 10) similar in both sexes: 12–15 longitudinal rows of 11–13 hooks of uniform length: 34–36 long ($\bar{x}$ = 35), posterior hooks spinelike. Anterior hooks more powerfully rooted than posterior hooks. Lemnisci obscured by eggs in most specimens, but 1 specimen has lemnisci that measure 10% of its body length (16.6 mm). The lemnisci are flat with 3 giant nuclei each and are not bound posteriorly to the inner body wall.

Male (Fig. 11a, b) (2 specimens): 95 mm and 98 mm long, 1.5 mm and 1.9 mm wide. Proboscis retracted in both specimens. Proboscis receptacle 1.2–1.3 mm long, 500 wide. Testes in tandem near posterior end. Anterior testes 5.6–6.8 mm long, 1 mm wide. Posterior testis 6.3 mm long, 1 mm wide. One specimen was broken at the level of the posterior testis, so that testis was not measured. Eight cement glands, each with single giant nucleus, located in compact group posterior to testes. Genital pore terminal.

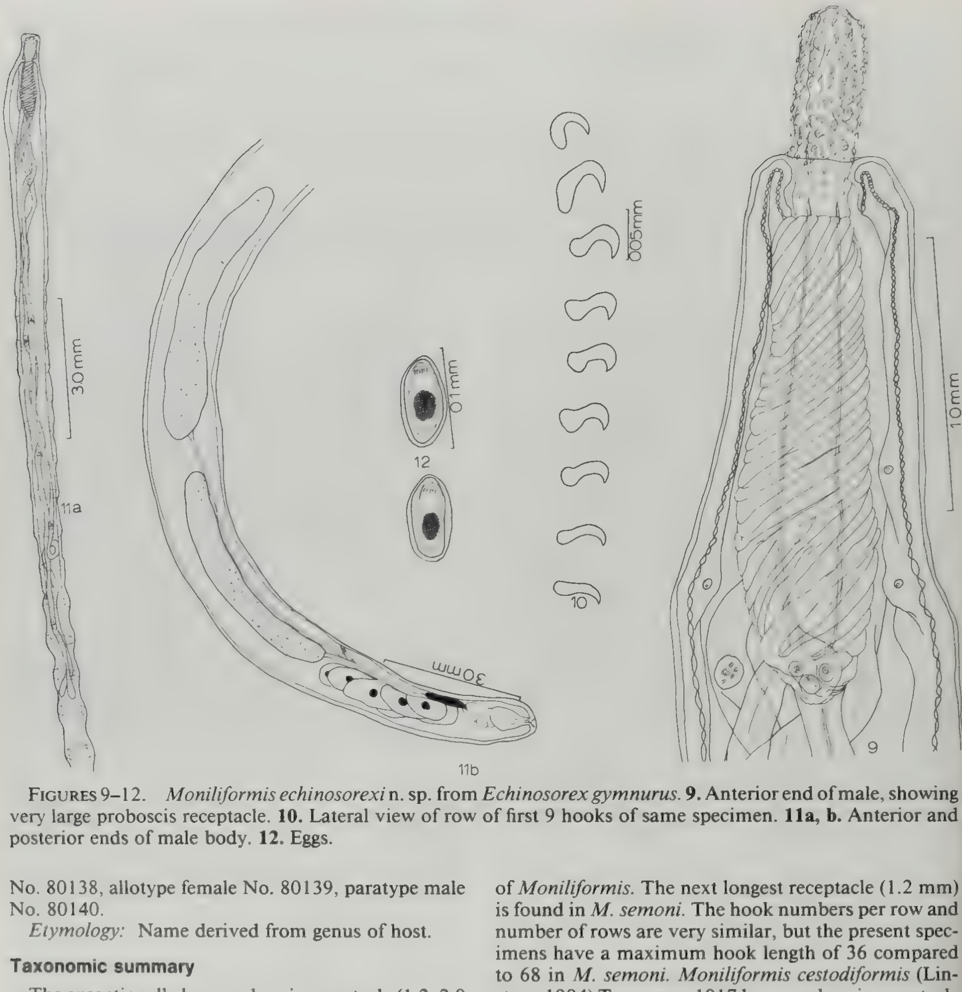
Female (6 specimens): 126–192 mm long ($\bar{x}$ = 156), 2.10–2.53 mm wide ($\bar{x}$ = 2.34). Proboscis (Fig. 9) 0.69–1.0 mm long ($\bar{x}$ = 0.78), 210–260 wide ($\bar{x}$ = 220). Proboscis receptacle 1.58–2.0 mm long ($\bar{x}$ = 1.84), 500–630 wide ($\bar{x}$ = 550). Uterine bell 1.40–1.50 mm ($\bar{x}$ = 1.45) from genital pore. Eggs (Fig. 12) measured through the body wall, 79–90 long ($\bar{x}$ = 86), 40 wide. Outer egg envelope sculptured.

Type host: Moon rat, *E. gymnurus* (Raffles).

Location: Small intestine.

Type locality: Ulu Gombak, Selangor, Peninsular Malaysia.

Type specimens: USNM Helm. Coll. holotype male



FIGURES 9–12. *Moniliformis echinosorexi* n. sp. from *Echinosorex gymnurus*. **9.** Anterior end of male, showing very large proboscis receptacle. **10.** Lateral view of row of first 9 hooks of same specimen. **11a, b.** Anterior and posterior ends of male body. **12.** Eggs.

No. 80138, allotype female No. 80139, paratype male No. 80140.

Etymology: Name derived from genus of host.

Taxonomic summary

The exceptionally long proboscis receptacle (1.2–2.0 mm) of this species differs greatly from all other species of *Moniliformis.* The next longest receptacle (1.2 mm) is found in *M. semoni.* The hook numbers per row and number of rows are very similar, but the present specimens have a maximum hook length of 36 compared to 68 in *M. semoni. Moniliformis cestodiformis* (Linstow, 1904) Travassos, 1917 has a proboscis receptacle 1.07 mm long and hook measurements almost equal

TABLE I. *New records of* Moniliformis moniliformis *from Malaysia.*

Host	Locality	Voucher specimens USNM Helm. Coll. No.
Moon rat, *Echinosorex gymnurus* (Raffles)	Bukit Legong, Kepong, Selangor	00000
Common tree shrew, [illegible]	Sungei Buloh, Selangor	80151
House rat, *Rattus rattus diardi** (Jentink)	Kota Baharu, Kelantan; Kuala Lumpur, Selangor	80146
Edward's rat, *Leopoldamys edwardsi* (Thomas)	Cameron Highlands, Pahang	80147
Long-tailed mountain rat, *Niviventer rapit* (Bonhote)	Cameron Highlands, Pahang	80148
Norway rat, *Rattus norvegicus* [illegible]	Kota Baharu, Kelantan	80149
Polynesian rat, *Rattus exulans* [illegible]	Kota Baharu, Kelantan	80144
Annandale's rat, *Rattus annandalei* (B[illegible]	Subang, Selangor	80145
Red-cheeked ground squirrel, *D*[illegible]	Cameron Highlands, Pahang	80150
Vordermann's flying squirrel, *Pet*[illegible]	Subang, Selangor	80153
Black banded squirrel, *Callosciurus* [illegible]	Ulu Langat, Selangor	80152

* New locality records only.

to the present specimens, but *M. cestodiformis* has 16–18 rows of 7–8 hooks each versus *M. echinosorexi* with 12–15 rows of 11–13 hooks.

One vial also contained a single female *M. moniliformis.* The proboscis has 14 rows of 8 hooks each and the receptacle is 700 long. No eggs were observed. The insectivorous diet of the host predisposes it to acanthocephalan infection so that chances of multispecific infections increase. Cross-fertilization between *M. moniliformis* and *M. echinosorexi* would not be expected because *M. moniliformis* has been observed by Buckner and Nickol (1975) not to hybridize with other members of the genus.

Moniliformis moniliformis (Bremser *in* Rudolphi, 1811)

Measurements of the remaining specimens of *Moniliformis* agreed with those of the type species, *M. moniliformis* as described by Van Cleave (1953), Petrochenko (1958), Yamaguti (1963), and Khairul Anuar (1977). Findings are summarized in Table I.

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METAZOAN PARASITES OF *HIMANTOPUS MEXICANUS* MULLER (AVES) FROM SOUTHWESTERN TEXAS, WITH A CHECKLIST OF HELMINTH PARASITES FROM NORTH AMERICA

Jose G. Hinojos and Albert G. Canaris
Department of Biological Sciences, The University of Texas at El Paso, El Paso, Texas 79968

ABSTRACT: Nineteen species of helminths were recovered from 34 of 35 black-necked stilts, *Himantopus mexicanus* Muller, collected from the Fort Bliss ponds, El Paso County, Texas. New host records are marked with an *. The species identified were: *Acoleus vaginatus, Davainea himantopodis, Diplophallus polymorphus,* **Eurycestus avoceti, Hymenolepis himantopodis, Hymenolepis* sp. 1, *Infula macrophallus, Cloacitrema michiganensis, Cyclocoelum lanceolatum, Notocotylus* sp., *Parastrigea mexicanus,* **Tanaisia fedtschenkoi, Capillaria* sp., **C. anatis,* **C. contorta,* **C. mergi,* **Chevreuxia americana, Eustronglydes mergorum,* and **Splendidofilaria* sp. Six species of mallophagan lice and 1 species of nasal mite, *Rhinonyssus himantopus,* were recovered.

Helminths showed little concentration for dominance (0.09), were not very evenly distributed (0.49 ± 0.08) nor very diverse (0.73 ± 0.14), and most species were highly aggregated. The helminth community consisted of an unusually large number of core species (10). Three large species of tapeworms exhibited mostly paired infections, were mutually exclusive, and were negatively associated (−1).

The family Recurvirostridae is comprised of stilts and avocets. They are cosmopolitan, medium-sized shore birds with long, slender bills that are straight or recurved (Johnsgard, 1981). The stilts, genus *Himantopus* Brisson, 1760, include approximately 10 species with representatives on all continents except Antarctica (Clements, 1974). *Himantopus mexicanus* Muller, the black-necked stilt, is found in North America and ranges from southwestern Canada and western and southeastern United States south through Mexico and Central America to Peru and Brazil (Blair et al., 1968). Black-necked stilts migrate along the Rio Grande Valley, U.S.A. Small populations utilize river edges, desert ponds, and man-made impoundments in the southwestern United States from April to September, and a few birds nest in this area. Black-necked stilts eat a wide range of foods, according to Pough (1953). Aquatic beetles, bugs, and fly larvae are staple foods, with snails and crustaceans less common in the diet. Young birds sometimes take considerable numbers of beetle and fly larvae (Johnsgard, 1981).

There have been some reports of parasites harbored by the black-necked stilt. McIntosh (1938) described a philopthalmid trematode, *Cloacitrema michiganensis,* from stilts in Florida, and Coil (1955) described a new species of cestode, *Infula macrophallus,* from a stilt collected in Oaxaca, Mexico. Burt (1978, 1980) recovered the tapeworm *Diplophallus polymorphus* Rudolphi, 1819, from both the black-necked stilt and the American avocet, *Recurvirostra americana* Gmelin, 1789, collected from several localities in the United States and discussed the taxonomic status of *D. polymorphus* and related tapeworms. Strandtmann (1951) described the nasal mite *Rhinonyssus himantopus* from *H. mexicanus* collected in Texas, and subsequently it was reported from Cuba and Louisiana (Cerny and Dusbabek, 1971; Pence, 1972).

This paper presents information about metazoan parasites recovered from *H. mexicanus* collected in southwestern Texas and compares our findings to those for the American avocet, the Eurasian stilt (*H. himantopus himantopus* L.), and 2 species collected in South Africa: *Himantopus himantopis meriodinalis* L. and *Recurvirostra avocetta* L. Observations and comments pertaining to exclusion among the cestodes also are given.

METHODS AND MATERIALS

Thirty-five black-necked stilts, *H. mexicanus*, were shot at the Fort Bliss ponds, El Paso County, Texas, from 10 May 1979 to 24 May 1984. Several juvenile American avocets and black-necked stilts were collected in the summer of 1984 from the same locality and examined only for the number and state of development of the 3 large species of tapeworms observed in the completed study. Birds were placed in individual plastic bags and those not examined within 2 hr were frozen and examined at a later date for metazoan parasites.

Trematodes and cestodes were fixed in alcohol–formalin–acetic acid (AFA), stained in Semichon's acid carmine, and mounted in Canada balsam or permount. Nematodes were fixed in 70% ethanol and cleared in temporary lactophenol mounts. Ectoparasites and na-

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sal mites were fixed in 70% ethanol, cleared in 10% KOH, and mounted in Lipshaw's mounting media.

Abundance data were used for most of the analyses. Standard measures of central tendency (means) and dispersion (variance, standard deviation, standard error), distributions (uniform, random, contagious), interspecific associations, correlation coefficients (r), and Spearman's rank coefficient for helminths were calculated with the aid of Microstat (Ecosoft, Inc., Indianapolis, Indiana) and Ecological Analysis—PC, Ecological Analysis Vol. 2—PC (Oakleaf Systems, Decorah, Iowa).

Clustering methods (group average with Euclidean distance, Euclidean distance squared, and Manhattan distance) for elucidation of helminth parasite community structure and for comparison to the helminth "core and satellite" species concept of Hanski (1982) as utilized for parasites by Bush and Holmes (1986a, 1986b) were done with the aid of Biostat II, A Multivariate Statistical Toolbox (Sigma Soft, Placentia, California). Helminth parasites from the black-necked stilt were classified into 4 groups based on their importance in the parasite community as determined by the second method (I_2) = percent prevalence × mean intensity. The groups were: (1) dominant species were those species characteristic of the community ($I > 1.0$); (2) codominant species were those contributing significantly to the community composition, but which do well in other habitats ($0.1 < I < 1.0$); (3) successful immigrant species were those species that occur infrequently and, although characteristic of the community, belong to another habitat ($0 < I < 0.1$); and (4) unsuccessful immigrant species were those species that gain access to the habitat but do not mature or reproduce, contribute little to the community, and were always characteristic of another community ($I = 0$). Data were also arranged in terms of their classification of importance in the helminth community using the I_2 method of Pence and Eason (1980). The I_2 results were used to help interpret clustering results and for comparison to the core–satellite community results.

When the variance/mean ratio for a species of helminth indicated its distribution was significantly contagious (clumped, aggregated), the degree of overdispersion was obtained by calculating the negative binomial parameter k, which is an inverse measure of the degree of overdispersion. Statistical significance was assumed when $P < 0.05$.

Simpson's index for concentration for dominance was calculated for helminths and ectoparasites, and Sorenson's index of similarity was used to compare helminth parasite faunas among *H. mexicanus, H. h. himantopus,* and *R. americana* according to the methods of Stone and Pence (1978). Mean diversity (H) and mean evenness (J), with confidence limits, were calculated for helminth parasites using Shannon's index as modified by Pielou (Zar, 1974).

Representative slides, numbers 78871–78880, have been deposited with the USNM Helm. Coll. API, USDA, BARC-East 1180, Beltsville, Maryland 20705.

RESULTS AND DISCUSSION

Of the 35 black-necked stilts, *H. mexicanus,* 97% were infected with at least 1 species of helminth parasite. Nineteen species of helminths were recovered: 7 Cestoidea, 5 Trematoda, and 7 Nematoda (Table I). No additional species of helminth parasites were observed after the 22nd bird was examined. Sixty-three percent of the hosts were infected with mallophagan lice and 6 species were recovered; 9% of the birds were infected with the nasal mite *Rhinonyssus himantopus* (Table I).

CESTOIDEA

Among the 3 groups of helminths, the cestodes showed the highest abundances. A pattern of infection was observed among the 3 largest species of cestodes. All *Diplophallus polymorphus, Acoleus vaginatus,* and *Infula macrophallus* inhabited the proximal one-third of the small intestine of the black-necked stilt and were mutually exclusive. They were mostly in pairs (*D. polymorphus* 89.5% of the time, *A. vaginatus,* 89.0%, and *I. macrophallus,* 50.0%). If not in pairs, the infections were predominantly of a single worm. In 1 instance, 3 *D. polymorphus* were recovered, but the sizes indicated they were immature. Burt (1978) also observed the occurrence of paired *Diplophallus polymorphus* from *R. americana* and *H. mexicanus* collected from 3 flyways in North America. Ahern and Schmidt (1976) noted this pattern of infection for *Diplophallus coili* from American avocets whereby 34 of 37 hosts had paired tapeworm infections. The occurrence of paired cestode infections in the American avocet was also observed by Garcia and Canaris (1987) from south-central Colorado and southwestern Texas. They reported that the cestode *D. coili* always occurred in pairs in the upper one-third of the small intestine and with a very high prevalence (93.9%). Ten black-winged stilts, *H. h. meriodinalis,* and 10 avocets, *R. avocetta,* collected from the southwestern Cape Province, South Africa, also showed this same pattern of paired infections for the 2 large species of tapeworms recovered, *A. vaginatus* and *D. polymorphus* (Canaris and Hinojos, pers. obs.). Ahern and Schmidt (1976) related the large size and pattern of infection of *D. coili* to competition for nutrients. They noted that in a more recent infection of 13 specimens, all were small and approximately the same size, but in an older infection of 16 specimens, 2 were larger than the others. This same pattern of multiple infections of a single species, *Diplophallus* sp. in both the black-necked stilt and American avocet and *In-*

TABLE I. *Parasites of the black-necked stilt,* Himantopus mexicanus, *from El Paso County, Texas.*

Birds examined	Number infected	Prevalence (%)	Intensity				Abundance		
			$\bar{x}$ ± SE		SD	Range	$\bar{x}$ ± SE		SD
Cestoidea (7)									
Hymenolepis himantopodis	26	74.3	42.8	9.8	47.9	1–253	31.8	7.6	45.2
Davainea himantopodis	25	71.4	159.8	43.7	218.6	4–845	114.1	33.4	197.7
Diplophallus polymorphus	19	54.3	2.0	0.08	0.3	1–3	1.1	0.2	1.0
Acoleus vaginatus	9	25.7	1.9	0.01	0.4	1–2	0.5	0.1	0.9
Hymenolepis sp. 1	8	22.9	9.5	3.5	9.8	1–31	2.2	1.0	6.0
Infula macrophallus	6	17.1	1.5	0.2	0.5	1–2	0.3	0.1	0.6
*Eurycestus avoceti**	1	2.9	—	—	—	—	0.9	0.9	5.2
Trematoda (5)									
Cyclocoelum lanceolatum	8	22.9	3.4	0.8	2.4	1–8	0.8	0.3	1.8
Parastrigea mexicanus	8	22.9	6.1	3.4	9.5	1–29	1.4	0.9	5.0
*Tanaisia fedtschenkoi**	3	8.6	19.3	8.0	13.9	4–31	1.7	1.1	6.4
Cloacitrema michiganensis	1	2.9	—	—	—	—	0.5	0.5	3.0
Notocotylus sp.	1	2.9	—	—	—	—	0.1	5.7	0.3
Nematoda (7)									
*Chevreuxia americana**	16	45.7	4.2	1.2	4.2	1–20	1.9	0.7	4.0
Capillaria sp.	10	28.6	2.6	0.7	2.6	1–7	0.7	0.3	1.7
*Capillaria contorta**	8	22.9	5.6	2.0	7.4	1–17	1.7	0.7	4.0
*Capillaria anatis**	6	17.1	4.5	1.8	6.2	2–12	1.0	0.5	2.9
*Capillaria mergi**	6	17.1	8.7	3.6	6.7	1–24	1.1	0.7	4.2
Eustronglydes mergorum	3	8.6	8.1	7.0	5.7	1–25	0.5	0.4	2.5
Splendidofilaria sp.*	1	2.9	—	—	—	—	—	—	—
Mallophaga (6)									
Quadraceps semifissus mexicanus	11	31.4	1.6	0.1	0.9	1–6	0.5	0.2	0.8
Actornithophilus mexicanus	8	22.9	1.9	0.2	0.9	1–3	0.4	0.4	1.0
Quadraceps hemichrons	6	17.1	1.0	0.1	0.4	1	0.2	0	0
Actornithophilus himantopi	3	8.6	2.0	0.1	0.6	1–3	0.2	0.7	1.2
Quadraceps semifissus semifissus	3	8.6	1.3	0.1	0.4	1–2	0.1	0.3	0.3
Austromenopon himantopi	2	5.7	1.5	0.1	0.4	1–2	0.1	0.5	0.7
Acarina (1)									
Rhinonyssus himantopus	3	8.6	5.0	2.0	3.5	3–9	0.4	0.3	1.7

* New host record.

fula macrophallus in the stilt, was observed in a sample of juveniles collected in El Paso County, Texas. Ahern and Schmidt further stated that as infections continued, the competition between worms was greater as they increased in size, hence only 2 worms survived. They also explained that the occurrence of more than a pair of large or average size tapeworms (80–280 mm long and 2.5–6.2 mm wide) might cause blockage, and further, that the nutrient requirements with more than 2 large tapeworms might decrease the chances for survival of both host and parasite. There are, then, at least 4 large species of tapeworms that showed predominantly paired infections in the upper one-third of the small intestine in adults of the Recurvirostridae: *A. vaginatus, D. coili, D. polymorphus,* and *I. macrophallus.*

According to the Association Analysis test, all possible pairings of the 3 large species of tapeworms in the black-necked stilt showed negative association (−1), and all were significant except *A. vaginatus* with *I. macrophallus.* It is not clear how these large tapeworm species are excluding each other, but they do appear to be limiting their own intensities by intraspecific competition.

A very unusual cestode, *Eurycestus avoceti* Clark, 1954, had been described and reported only from the American avocet from Nebraska (Clark, 1954). Ours was the second recovery of this tapeworm. The strobila consisted of extremely short, but very wide, proglottids, and a functional scolex was absent. These worms may be overlooked easily because of their nonworm-like appearance and small size.

TREMATODA

Five species of trematodes were collected (Table I). *Cyclocoelum lanceolatum* Wedl, 1858, and *Parastrigea mexicanus* Coil, 1957, had the highest prevalences. *Parastrigea mexicanus* was described from the American avocet from the Gulf of Mexico by Coil (1957) and was also reported from the black-necked stilt in Cuba (Dubois and Macko, 1972). *Tanaisia fedtschenkoi* Byrd and

Denton, 1950, had the highest mean intensity (19.3). This trematode has been reported as a parasite of the genitourinary tract of many aquatic and semiaquatic birds including the common snipe *Capella gallinago* L. collected from southwestern Texas and south-central Colorado (Leyva et al., 1980). Only 1 stilt was infected with *Cloacitrema michiganensis* McIntosh, 1938, and a total of 18 specimens was recovered from the small intestine. *Cloacitrema michiganensis* was originally described from specimens recovered from the black-necked stilt and spotted sandpiper *Actitis macularia* L. from Florida and Michigan, respectively (McIntosh, 1938). This is the first observation of this parasite from a stilt collected in the southwestern U.S.A.

NEMATODA

Seven species of nematodes were recovered (Table I). The nematode *Chevreuxia americana* Schmidt, 1968, had the highest abundance (1.9) and is a new host record. This nematode was described from an American avocet in Colorado by Schmidt (1968), and was recovered under the koilon of the gizzard. The black-necked stilt's infections were also under the koilon. The genus *Capillaria* was represented by 4 species: *Capillaria* sp., *C. contorta, C. anatis,* and *C. mergi,* and these were new host records. One specimen of *Splendidofilaria* sp. was recovered from the subdermal fascia in the neck region, and this was a new host record.

MALLOPHAGA

Six species of mallophagan lice were recovered, and all 6 have previously been reported from the black-necked stilt (Table I). *Quadraceps semifissus mexicanus* had the highest abundance (0.5) followed by *Actornithophilus mexicanus* (0.4).

COMMUNITY STRUCTURE

The helminth parasite fauna, by Simpson's index, showed little concentration for dominance (0.09). The index was higher for the mallophagan ectoparasites (0.22), with *Actornithophilus mexicanus* and *Quadraceps semifissus mexicanus* contributing the most to the concentration for dominance. Shannon's test for mean diversity (H) and evenness (J) indicated that the helminth parasites were not very diverse (0.73 $\pm$ 0.14) and not very evenly distributed among the hosts (0.49 $\pm$ 0.08).

Sorenson's index of similarity indicated the black-necked stilt's helminth fauna was more similar to that of the American avocet (27.9%) than to that of the Eurasian black-winged stilt (22.5%), and the American avocet's helminth fauna was more similar to its North American relative, the black-necked stilt, than to the Eurasian black-winged stilt (12.7%). Comparison could not be made to other species of stilts in the family Recurvirostridae because not enough information about their helminth parasite faunas has been reported.

Thirteen of the 14 most prevalent helminths were significantly contagious (clumped, aggregated) according to the variance to mean ratio, and *k* values for these were low, indicating a high degree of overdispersion (Table III).

In terms of classification of importance in the helminth parasite community, there were 10 dominant, 5 codominant, no successful immigrant, and 4 unsuccessful immigrant species (Table II). All of the dominant species from the I_2 method also included all the core species from the clustering methods (Table II).

Clustering techniques indicated there were 10 core and 9 satellite species (Table II). Community structure for helminths from the black-

TABLE II. *Classification and importance values (I_2) of helminths from the black-necked stilt,* Himantopus mexicanus, *from El Paso County, Texas.*

Helminth species	I_2 values
Dominant species*	
Davainea himantopodis	114.10
Hymenolepis himantopodis	31.80
Hymenolepis sp. 1	2.18
Chevreuxia americana	1.91
Capillaria contorta	1.69
Tanaisia fedtschenkoi	1.66
Parastrigea mexicanus	1.40
Capillaria mergi	1.14
Diplophallus polymorphus	1.09
Capillaria anatis	1.06
Codominant species†	
Cyclocoelum lanceolatum	0.77
Capillaria sp.	0.74
Eustronglydes mergorum	0.49
Acoleus vaginatus	0.46
Infula macrophallus	0.26
Successful immigrant species	
None	
Unsuccessful immigrant species†	
Cloacitrema michiganensis	0
Eurycestus avoceti	0
Notocotylus sp.	0
Splendidofilaria sp.	0

* Core species.
† Satellite species.

TABLE III. *Overdispersion analysis and measure of degree of aggregation in the 14 most prevalent helminths from black-necked stilts,* Himantopus mexicanus, *collected in El Paso County, Texas.*

Helminth species	Variance/ mean ratio	Significantly clumped	*k*
Davainea himantopodis	342.5*	†	0.22
Hymenolepis himantopodis	64.3*	†	0.38
Tanaisia fedtschenkoi	25.0*	†	0.02
Parastrigea mexicanus	18.1*	†	0.09
Hymenolepis sp. 1	16.6*	†	0.08
Eustronglydes mergorum	13.2*	†	0.03
Capillaria contorta	9.7*	†	0.09
Chevreuxia americana	8.2*	†	0.30
Capillaria anatis	8.0*	†	0.07
Cyclocoelum lanceolatum	4.2*	†	0.36
Capillaria sp.	3.9*	†	0.09
Acoleus vaginatus	1.5*	†	0.43
Infula macrophallus	1.5*	†	0.36
Diplophallus polymorphus	1.0	Uniform distribution	

* Variance to mean ratio significantly greater than 1.
† Significant.

necked stilt was more similar to that of the more closely related American avocet, *Recurvirostra americana,* from El Paso County, Texas (Garcia and Canaris, 1987), than to that of the common snipe, *Capella gallinago,* from the same locality (Leyva et al., 1980). Nineteen species of helminths were recovered from both the stilt and avocet, but only 14 from the common snipe. Three (21%) of the helminths from the snipe were considered to be core species as compared to 10 (53%) and 8 (42%), respectively, for the stilt and avocet. Out of 38 species of helminths recovered from the stilt and avocet, 4 were common to both birds, and 3 of these were core species if *D. polymorphus* and *D. coili* are considered synonymous (Burt, 1978, 1980). The stilt shared 2 species of helminths, *Capillaria contorta* and *Tanaisia fedtschenkoi,* with the snipe. They were core species in the stilt and a satellite and core species in the snipe. The avocet shared 1 species with the snipe, the ubiquitous *Echinostomum revolutum,* which was a satellite species in both species of birds (Garcia and Canaris, 1987).

Apparently in southwestern Texas, there is a much larger number of species of helminths better adapted to the stilt and avocet than to the snipe. Snipe in southwestern Texas are transitory migrants and absent in the summer. Both stilt and avocet may linger during migration and both species nest in southwestern Texas; therefore, the opportunity to establish infective pools of helminths in this locality are more likely for these 2 species of recurvirostrids.

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TABLE IV. *Checklist of helminth parasites of the black-necked stilt,* Himantopus mexicanus, *from North America.*

Parasite group	Geographic region	Citation
Trematoda (6)		
Cloacitrema michiganensis	Florida	McIntosh, 1938
Hofmonostomum himantopodis	Puerto Rico	Yamaguti, 1971
Notocotylus sp.	Texas	This study
Parastrigea mexicanus	Cuba	Dubois and Macko, 1972
Tanaisia fedtschenkoi	Texas	This study
Zygocotyle lunata	U.S.A.	Yamaguti, 1971
Cestoidea (7)		
Acoleus vaginatus	U.S.A.	Yamaguti, 1959
Davainea himantopodis	Texas	This study
Diplophallus polymorphus	N. America	Burt, 1978
Eurycestus avoceti	Texas	This study
Hymenolepis himantopodis	Texas	This study
Hymenolepis sp.	Texas	This study
Infula macrophallus	Mexico and Texas	Coil, 1955, 1963, 1968
Nematoda (10)		
Capillaria anatis	Texas	This study
Capillaria contorta	Texas	This study
Capillaria mergi	Texas	This study
Capillaria obsignata	Cuba	Barus and Hernandez, 1971
Capillaria sp	Texas	This study
Chevreuxia americana	Texas	This study
Chevreuxia revolta	Cuba	Barus and Hernandez, 1971
Eustrongylides [illegible]	Texas	This study
Splendidofilaria	Texas	This study
Tropisurus nouveli	Cuba	Barus and Hernandez, 1971

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WAKUBITINEMA TOYAMAI N. GEN. AND N. SP. (NEMATODA: SEURATOIDEA: QUIMPERIIDAE) FROM THE INTESTINE OF *RANA* (*LIMNONECTES*) *NAMIYEI* (AMPHIBIA: RANIDAE) ON OKINAWA ISLAND, JAPAN

Hideo Hasegawa
Department of Parasitology, School of Medicine, University of the Ryukyus, Nishihara, Okinawa, 903-01, Japan

ABSTRACT: *Wakubitinema* n. gen. (Nematoda: Seuratoidea: Quimperiidae: Quimperiinae) is erected for *Wakubitinema toyamai* n. sp. from the small intestine of *Rana* (*Limnonectes*) *namiyei* Stejneger, 1901, on Okinawa Island, Japan. *Wakubitinema* resembles *Paraquimperia* Baylis, 1934, and *Desmognathinema* Baker et al., 1987, but is readily distinguished from the former genus by the distinctly divided esophagus and the absence of cervical flanges and lateral alae, and from the latter genus by the postesophageal position of the excretory pore and cervical papillae and the presence of preanal unpaired papilla in males. Close morphological similarities between *Wakubitinema* and *Paraquimperia* may suggest that *Wakubitinema* has evolved from a quimperiid fish.

The superfamily Seuratoidea is composed of numerous archaic genera that link the Cosmocercoidea and the advanced Ascaridida or the Spirurida (Chabaud et al., 1960; Chabaud, 1978). Chabaud (1978) pointed out that the Kathlaniidae is intermediate between Cosmocercoidea and certain Seuratoidea, and Baker (1980) presented a hypothesis on the evolutionary line through Kathlaniinae (Cosmocercoidea: Kathlaniidae) to Quimperiidae (Seuratoidea). However, the evolutionary relationship within Quimperiidae has not been adequately elucidated. During a survey on the parasitic helminths of relict frogs on Okinawa Island, Japan, a quimperiid nematode closely related to the genus *Paraquimperia*, parasitic in eels, was recovered. A new genus is proposed for this parasite and its phylogenic relationship with other quimperiids is discussed in this paper.

MATERIALS AND METHODS

The frogs were collected by hand in the mountainous jungles of Kunigami-son, Okinawa, Japan, from 1983 to 1984. Frogs were killed by ether inhalation and examined for parasites. Nematodes were fixed in hot 70% ethanol, cleared in glycerin alcohol solution, and mounted on slides with 50% glycerin. Figures were made with the aid of a drawing tube, Olympus BH-DA-LB. Measurements given are for the holotype male and allotype female, followed in parentheses by range for paratype males and females.

DESCRIPTION

Wakubitinema n. gen.

Seuratoidea, Quimperiidae, Quimperiinae. Cervical flanges and lateral alae absent. Cephalic papillae and amphids pedunculate. Mouth triangular. Buccal cavity poorly developed; pharynx short and with 3 pharyngeal teeth anteriorly. Esophagus distinctly divided into anterior muscular and posterior glandular portions. Intestinal cecum absent. Nerve ring at midlevel of muscular portion of esophagus. Excretory pore behind posterior end of esophagus. Excretory system symmetrical. Cervical papillae minute. Spicules alate. Gubernaculum present. Caudal papillae sessile. Preanal unpaired papilla present. Oblique muscle bands present in preanal region. Preanal sucker and caudal alae absent. Vulva in posterior one-third of body. Vagina short, directing anteriorly. Amphidelphic. Parasitic in small intestine of amphibian.

Type and only species: Wakubitinema toyamai.

Wakubitinema toyamai n. sp.
(Figs. 1–10)

General: With characters of the genus as outlined above. Worm of medium size; transparent when alive. Body cylindrical, tapered in both extremities (Figs. 1, 2, 5, 9); soft and delicate. Cuticle thin and with faint transverse striations. Outer circle of cephalic papillae composed of 2 subdorsal, 2 subventral, and 1 pair of lateral papillae; inner circle composed of 6 long papillae; amphids set closely to lateral papillae of outer circle (Figs. 3, 4). Muscular portion of esophagus thinner but longer than glandular portion; middle of glandular portion slightly constricted (Figs. 1, 2). Lateral canals extending within lateral chords along almost whole length of worm (Fig. 2); excretory vesicle thin-walled, elliptical in ventral view, and surrounded by large glandular tissue containing a few large nuclei and extending on subventral sides of body from level of glandular esophagus to anterior portion of intestine (Figs. 1, 2). Cervical papillae at level between posterior end of esophagus and excretory pore (Figs. 1, 2).

Male (holotype and 12 paratypes): Body 14.0 (12.4–17.6) mm long and 0.19 (0.21–0.27) mm wide at midbody. Pharynx 43 (39–50) μm long; muscular portion of esophagus 0.50 (0.45–0.50) mm long by 53 (49–63) μm wide; glandular portion of esophagus 0.40 (0.39–0.45) mm long by 103 (96–135) μm wide. Nerve ring 0.29 (0.29–0.36) mm and excretory pore 1.31 (1.11–

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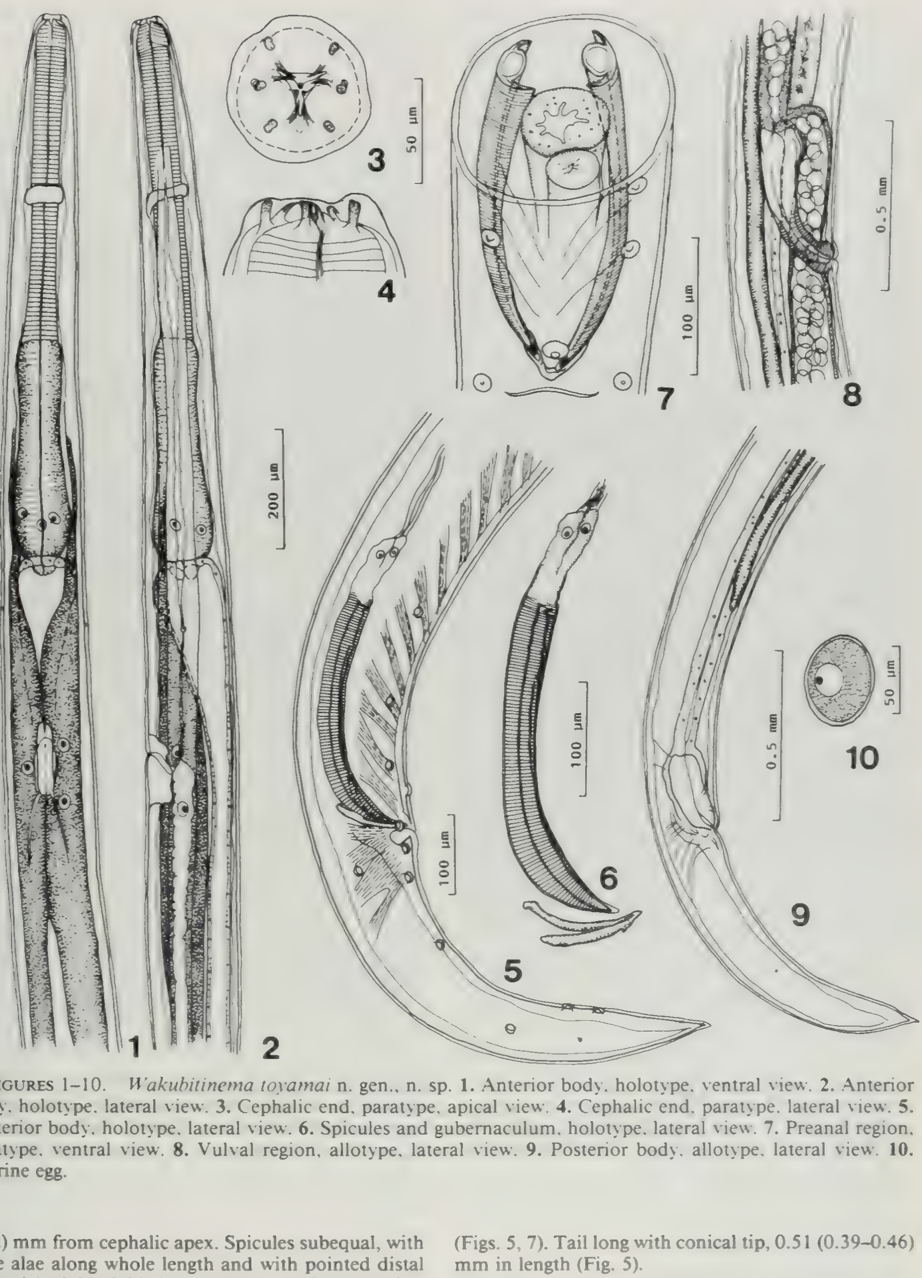

FIGURES 1–10. *Wakubitinema toyamai* n. gen., n. sp. **1.** Anterior body, holotype, ventral view. **2.** Anterior body, holotype, lateral view. **3.** Cephalic end, paratype, apical view. **4.** Cephalic end, paratype, lateral view. **5.** Posterior body, holotype, lateral view. **6.** Spicules and gubernaculum, holotype, lateral view. **7.** Preanal region, paratype, ventral view. **8.** Vulval region, allotype, lateral view. **9.** Posterior body, allotype, lateral view. **10.** Uterine egg.

1.42) mm from cephalic apex. Spicules subequal, with wide alae along whole length and with pointed distal ends: right 0.28 (0.23–0.39) mm long; left 0.31 (0.25–0.40) mm long (Figs. 5–7). Gubernaculum bifurcate, 105 (68–105) μm long (Figs. 5–7). Ten or 11 pairs of sessile caudal papillae present: 3 pairs preanal; 1 adanal and 6 or 7 postanal: second (or third) and fifth pairs of postanal papillae situated laterally (Fig. 5). A single papilla present on midventral part of preanal region (Figs. 5, 7). Tail long with conical tip, 0.51 (0.39–0.46) mm in length (Fig. 5).

Female (allotype and 18 paratypes): Body 20.8 (13.6–28.5) mm long and 0.33 (0.21–0.39) mm wide at midbody. Pharynx 55 (43–63) μm long; muscular portion of esophagus 0.57 (0.49–0.63) mm long by 68 (58–95) μm wide; glandular portion of esophagus 0.44 (0.36–0.50) mm long by 133 (107–158) μm wide. Nerve ring 0.38 (0.31–0.38) mm and excretory pore 1.48 (1.11–

1.75) mm from cephalic apex. Vulva 14.4 (9.42–19.7) mm from cephalic apex and slightly elevated (Fig. 8). Anterior oviduct flexed posteriorly, then flexed again forming a loop at level anterior to vulva and running anteriorly to join ovary; ovary very long, directed posteriorly extending to preanal region (Fig. 9). Posterior oviduct flexed anteriorly and then turned posteriorly forming a loop at level posterior to vulva and running posteriorly to connect with ovary; ovary directed anteriorly extending to level of loop of anterior oviduct. Tail long, tip conical, 0.91 (0.65–1.11) mm in length (Fig. 9).

Eggs: Uterine eggs elliptical, thin-shelled, containing 1- to 2-cell stage embryo, 56–73 by 43–52 μm (Fig. 10).

Taxonomic summary

Diagnosis: The genus *Wakubitinema* is closest to *Paraquimperia* Baylis, 1934 (Quimperiinae), in having a small buccal cavity, pharyngeal teeth, distinct oblique musculature at posterior part of the male, and an unpaired preanal papilla in male. However, *Wakubitinema* is readily distinguished from *Paraquimperia* by the distinctly divided esophagus and the absence of cervical flanges and lateral alae (Moravec, 1966). *Wakubitinema* also resembles *Desmognathinema* Baker et al., 1987 in that both possess a divided esophagus, oblique musculature in the preanal portion, and lack cervical and lateral alae and a preanal sucker. Nevertheless, *Wakubitinema* differs clearly from *Desmognathinema* in that the latter has an excretory pore and deirids at the junction of the 2 portions of esophagus, and lacks pharyngeal teeth and a preanal unpaired papilla in the male (Baker et al., 1987). *Wakubitinema* is also related to *Pseudohaplonema* Wang et al., 1978, from freshwater turtles of China, in having a divided esophagus and in lacking lateral alae and a preanal sucker in the male. Although the description of *Pseudohaplonema* is somewhat obscure, *Wakubitinema* may be distinguished from this genus of which glandular esophagus is about 3–4 times the length of muscular esophagus, and the nerve ring is situated at the posterior end of the muscular esophagus (Wang et al., 1978).

Specimens deposited: Holotype male, USNM Helm. Coll. No. 79846; allotype female, USNM Helm. Coll. No. 79847; paratypes, National Science Museum, Tokyo, Japan, NSMT As-98765.

Host: Rana (*Limnonectes*) *namiyei* Stejneger, 1901.

Locality: Kunigami-son, Okinawa Island, Japan.

Site in host: Small intestine.

Date of collection: 16 January 1984 (holotype and 2 paratypes); 2 June 1984 (allotype and 10 paratypes); 14 September 1984 (18 paratypes).

Etymology: The generic name is derived from the Okinawan name of the frog, "Wakubichi," and the species name is dedicated to Mr. Masanao Toyama, Okinawa Herpetological Society.

Ecological notes: Wakubitinema toyamai was recovered from 6 out of 8 *R.* (*L.*) *namiyei* collected in streams: in winter (2/2), summer (1/1), and autumn (3/5); intensity of infection was 1 to 16 (mean 8.8). This nematode was not detected from other frog species examined in the same locality: 40 *Rana narina* all gathered in streams for breeding in winter; 4 *Rana ishikawae* found on banks of the streams (2 in winter and 2 in summer); 2 *Rana holsti* on the stream banks in summer; 12 *Rhacophorus viridis viridis* on trees and grasses near the streams (10 in winter and 2 in summer).

DISCUSSION

Baker (1980) suggested that the evolutionary line towards the Quimperiidae has arisen from *Falcaustra* (*Spironoura*) Leidy, 1856 (Cosmocercoidea: Kathlaniidae: Kathlaniinae), and may be traced through the genus *Megalobatrachonema* Yamaguti, 1941, which shows clear affinities to the Quimperiidae. The presence of the preanal sucker or pseudosucker in *Falcaustra* and in some *Megalobatrachonema* may indicate that these structures are archaic. It is therefore supposed that *Haplonema* Ward and Magath, 1917, *Paraquimperia* Baylis, 1934, *Paraseuratum* Johnston and Mawson, 1940, *Pseudohaplonema, Desmognathinema,* and *Wakubitinema* are advanced genera because the preanal sucker is absent or greatly reduced (cf. Chabaud, 1978; Wang et al., 1978; Baker et al., 1987). On the other hand, the wide cervical flange, which is found in *Haplonema, Quimperia* Gendre, 1928, *Paraquimperia, Paragendria* Baylis, 1939, and *Ezonema* Boyce, 1971 (cf. Chabaud, 1978), seems to be a characteristic developed in quimperiids adapted to fishes.

Baker et al. (1987) noted that *Desmognathinema,* a parasite of North American salamanders, most resembles *Quimperia.* However, *Desmognathinema* is also close to *Paraquimperia,* because in the latter genus the preanal sucker or pseudosucker is absent (present in *Quimperia*) and the esophagus is divided in some representatives such as *P. anguillae* Karve, 1944 (Moravec, 1966). Morphological resemblance among *Paraquimperia, Desmognathinema,* and *Wakubitinema* suggests that they have derived from a common ancestor. Apparently, *Wakubitinema* is a "captured parasite" (*sensu* Chabaud, 1981) in an aquatic environment as in the case of *Desmognathinema* (Baker et al., 1987), and its ancestor might be a fish parasite close to *Paraquimperia.* Probably, the cervical flanges were reduced during adaptation to the amphibian intestine.

The host, *Rana* (*Limnonectes*) *namiyei,* is distributed only in the mountainous areas of Okinawa Island, Japan (Frost, 1985), and is considered to be a relict form. Because *Wakubitinema toyamai* was detected only in *R.* (*L.*) *namiyei* among frogs examined at the same locality, it seems likely that this parasite is host specific to *R.* (*L.*) *namiyei.* Baker (1983) considered that

none of the nematode groups occurring mainly in frogs are host specific. However, it is probable that the ecological and behavioral differences among frog species result in some degree of host specificity. *Rana* (*L.*) *namiyei* is the most aquatic anuran species in the localities studied, spending considerable time in streams, and is often observed preying upon crabs. These habits differ from those of more terrestrial Okinawan frogs, which have relatively limited contact with stream water except during the spawning season. Also they feed primarily on terrestrial insects. Although the life histories of quimperiid nematodes have not been fully elucidated, it has been demonstrated that aquatic invertebrates may play roles as intermediate or paratenic hosts (Bain and Philippon, 1969; Vassiliadès, 1972; Moravec, 1974). The host specificity of *W. toyamai* for a primarily aquatic frog species tends further to support this contention that aquatic invertebrates are necessary for the transmission of quimperiid nematodes of amphibians. Sampling for parasites in the frog community when most species return to the streams for breeding purposes would be invaluable in determining whether there is an evolutionary or ecological basis for host specificity in this host–parasite system.

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HELMINTHS OF SWALLOWS OF THE MOUNTAINS OF COLORADO, INCLUDING *ACUARIA COLORADENSIS* N. SP. (NEMATODA: SPIRURATA)

Frank J. Sherwin and Gerald D. Schmidt*
Department of Biological Sciences, University of Northern Colorado, Greeley, Colorado 80639

ABSTRACT: Thirty-three tree swallows, *Iridoprocne bicolor,* and 5 violet-green swallows, *Tachycineta thalassina,* from the eastern slope of the Rocky Mountains of northern Colorado were examined for helminths. Six species were found: *Diplotriaena obtusa, Acuaria coloradensis* n. sp. (Nematoda); *Vitta magniuncinata, Angularella audubonensis* (Cestoda); *Plagiorchis maculosus* (Trematoda); *Mediorhynchus papillosus* (Acanthocephala). The new species is most similar to *A. multispinosa* Perez Vigueras, 1937, but has 11 pairs of anal papillae and spicules measuring 125 μm and 140 μm.

Although many reports have been published on helminths of various species of swallows (Passeriformes: Hirundinidae) in many parts of the world, there are few records for North America. The only records from the North American Rocky Mountain region are those of Kayton and Schmidt (1975) and Stamper and Schmidt (1984), who studied the cliff swallow, *Petrochelidon pyrrhonota,* and Stabler and Kitzmiller (1970), who found *Microfilaria* sp. in the violet-green swallow in Colorado. Because we have found few reports of helminths from the tree swallow, *Iridoprocne bicolor,* and the violet-green swallow, *Tachycineta thalassina,* we examined several specimens of each.

MATERIALS AND METHODS

Thirty-three tree swallows were collected from May to August 1985 near Red Feather Lakes, Colorado, in the Montaine Zone, approximately 2,700 m above sea level. Five of the more rare violet-green swallows were shot near Bailey, Colorado, at about 3,000 m above sea level. Worms were prepared by conventional techniques as described by Kayton and Schmidt (1975). Measurements are in microns unless otherwise indicated.

RESULTS

One hundred ninety-two helminths were recovered, representing 7 species (Table I). All are new host and locality records. One new species is represented by a single complete male and 2 female fragments. The following description is based on these specimens.

Acuaria coloradensis n. sp.
(Figs. 1–5)

Spirurata, Acuariidae. Small, delicate worms with the characters of the genus as stated by Skrjabin et al. (1965). Cordons spiny (Fig. 1). Two pseudolabia bilateral to mouth. Nerve ring posterior to junction of pharynx and muscular esophagus. Muscular esophagus slender, glandular esophagus long.

Male (Figs. 2, 5): 7.24 mm long, 164 greatest width. Cordons (Figs. 1, 4) 1.39 to 1.44 mm long. Nerve ring and deirids 190 from anterior end. Pharynx 145, muscular esophagus 395, glandular esophagus 1.58 mm long. Tail (Fig. 5) slightly coiled, with slender alae. Caudal papillae symmetrically arranged as follows: 4 preanal pairs in a 1 + 3 arrangement; 7 postanal pairs in a 3 + 4 arrangement. Spicules subequal; left spicule 140 long, right spicule 125 long, both with simple rounded tip.

Female (1 gravid piece available): 191 wide, anus 2.35 mm from posterior end (Fig. 3). Eggs 35–36 by 23–24.

Taxonomic summary

Of the 70 known species of *Acuaria* only 5 are known to have spinous cordons. *Acuaria coloradensis* is easily differentiated from all of these by the anal papillae, which are arranged in 11 pairs; the other 5 species have no more than 10 pairs. Of those *Acuaria* with spinous cordons, *A. coloradensis* is most similar to *A. multispinosa* (Perez Vigueras, 1938) Skrjabin, Sobolev, et Ivashkin, 1965, from a bittern in Cuba, having similar lengths of cordons, muscular esophagus, and glandular esophagus in the male. In male *A. multispinosa* the cordons are 1.92 mm, the muscular esophagus 560, and glandular esophagus 1.7 mm long. More important differences between the 2 species are the number and arrangement of the anal papillae, and spicule lengths. *Acuaria coloradensis* has 11 pairs of anal papillae and spicules measuring 125 and 140, while *A. multispinosa* has 9 pairs of anal papillae and a single papilla located ventromedially near the tip of the tail. The spicule lengths of *A. multispinosa* are 220 and 740.

Of the remaining species of *Acuaria, A. coloradensis* is most similar to *A. anthuris* (Rudolphi, 1819) Railliet, Henry, et Sisoff, 1912, from passerine birds in Europe and Asia. Both species have similar body size and shape,

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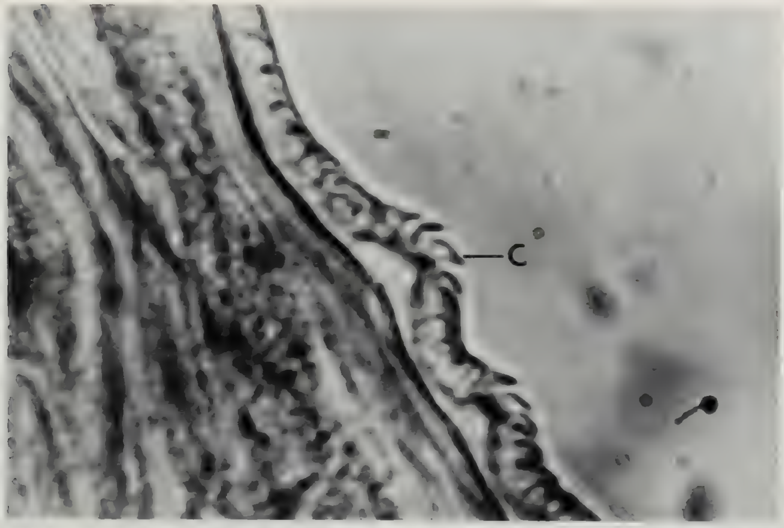

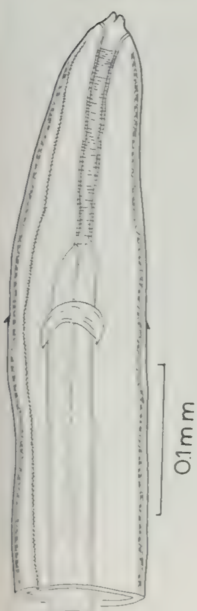

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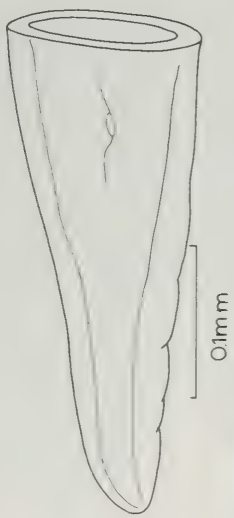

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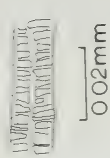

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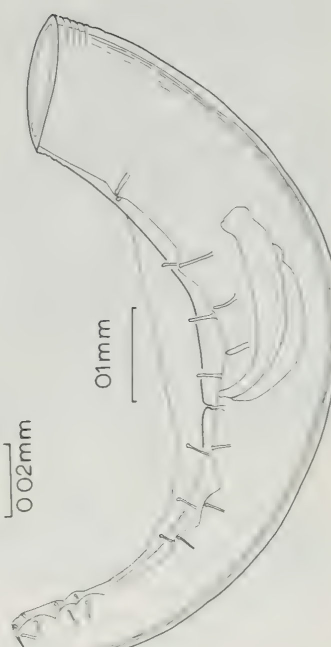

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FIGURES 1–5. *Acuaria coloradensis* n. sp. from *Tachycineta thalassina* in Colorado, U.S.A. **1.** Lateral view of cordon, showing its spiny nature. **2.** Dorsal view of anterior end. **3.** Ventral view of tail of allotype female, showing location of anus. **4.** Dorsal view of small part of cordon. **5.** Lateral view of holotype male.

TABLE I. *Helminths recovered from 2 species of swallows in Colorado.*

Host and parasite	Location	Prevalence	Intensity	Voucher specimens USNM Helm. Coll. No.
Tree swallow, *Iridoprocne bicolor*				
Angularella audubonensis Stamper et Schmidt, 1984	Small intestine	7/33	1–4	79506
Vitta magniuncinata Burt, 1938	Small intestine	3/33	1	79507
Plagiorchis maculosus Braun, 1901	Small intestine	24/33	1–17	79508
Diplotriaena obtusa Henry et Ozoux, 1909	Abdominal air sacs	16/33	1–6	79504
Mediorhynchus papillosus Van Cleave, 1916	Small intestine	2/33	1	79505
Violet-green swallow, *Tachycineta thalassina*				
Acuaria coloradensis n. sp.	Under gizzard koilon	1/5	3	–
Angularella sp.	Small intestine	1/5	3	79509

approximately the same cordon lengths, and anal papilla arrangement. However, *A. anthuris* does not have spiny cordons, and has spicules 200–240 and 260–288 long.

Type host: *Tachycineta thalassina* (Swainson, 1827) Cabanis, 1851.

Location: Under the koilon of the ventriculus.

Type locality: Bailey, Park County, Colorado, U.S.A

Etymology: Named for the state of Colorado.

Type specimens: U.S.N.M. Helm. Coll. holotype male No. 79861, allotype female (fragment), No. 79862, paratype male (fragment) No. 79683.

DISCUSSION

None of the parasites reported in this study has been reported from these hosts before. The only earlier reports of helminths we have found are: (*Iridoprocne bicolor*) *Physocephalus* sp., by Krahwinkel and McCue (1967); *Plagiorchis noblei* (experimental), by Blankespoor (1975); *Oxyspirura petrowi*, by Pence (1972); *Microfilaria* sp., by Greiner et al. (1975) and Clark et al. (1968); (*Tachycineta thalassina*) *Microfilaria* sp., by Greiner et al. (1975). Obviously more extensive surveys, especially of the violet-green swallow, would yield interesting results. However, no more birds were available to us.

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HYPOECHINORHYNCHUS THERMACERI N. SP. (ACANTHOCEPHALA: HYPOECHINORHYNCHIDAE) FROM THE DEEP-SEA ZOARCID FISH *THERMACERES ANDERSONI* ROSENBLATT AND COHEN, 1986

I. de Buron
Laboratoire de Parasitologie Comparée, UA 698 CNRS, USTL, Place E. Bataillon, 34060 Montpellier Cedex, France

ABSTRACT: *Hypoechinorhynchus thermaceri* n. sp. is described, based on 2 male and 5 nongravid female specimens collected from *Thermaceres andersoni* (Zoarcidae). This is the second record of an acanthocephalan parasite of an abyssal fish. The new species differs from *H. alaeopis* and *H. magellanicus* in the number of longitudinal rows of proboscis hooks, the total number of proboscis hooks, and the size of the various types of hooks. *Hypoechinorhynchus alaeopis* has been found in the subantarctic region and in the western Pacific, *H. magellanicus* in the subantarctic, and *H. thermaceri* is reported from the eastern Pacific.

During March 1984, the French expedition BIOCARYSE conducted a survey of the biota of the hydrothermal vents on the East Pacific Rise (103°W, 13°N) (Pacific Ocean). As a part of this effort, specimens of zoarcid fish were captured in bottom traps (depth, 2,600 m) laid by the submersible *Cyana*. Studies on the helminth parasites revealed, among others, specimens of an acanthocephalan that represent a new species.

MATERIALS AND METHODS

Intestines of the eelpout, *Thermaceres andersoni* Rosenblatt and Cohen, 1986, were preserved in methanol aboard ship and brought to the laboratory for examination. Specimens of the acanthocephalan described herein were removed from the small intestine, dehydrated in an alcohol series, and mounted in balsam. Two of the specimens were stained with acetocarmine and 1 male was dissected in order to study its genitalia in detail.

All measurements were made with a micrometre slide, and, unless noted otherwise, are given as means in micrometres, with the ranges in parentheses. The proboscis lengths given are exclusive of the neck and the proboscal hooks were only measured in profile. The widths presented represent maximum measurements. Length of the male reproductive system is presented as percentage of the trunk length.

DESCRIPTION

The following is the description of a new species of *Hypoechinorhynchus* Yamaguti, 1939, based on 2 male (1 dissected) and 5 nongravid female specimens.

Hypoechinorhynchus thermaceri n. sp.

General: Trunk unarmed. Cuticle thick. Cerebral ganglion at posterior end of double-walled proboscis receptacle. Neck short. Proboscis armed with 11–12 rows of 3 hooks each; 1 large (C I) with root (R) and 2 smaller (C II, C III) rootless spines.

Male: Trunk 3.5 mm long by 0.45 mm wide (Fig. 2). Proboscis could not be measured accurately because of mounted position. Hooks in 12 rows of 3 each with respective lengths of: C I = 85, C II = 50, C III = 40 (Fig. 3). Reproductive system length/trunk length 65%; with 6 cement glands. Safftigen's pouch large. Two testes tandemly arranged. Anterior testis 480 long by 180 wide; posterior testis 400 long by 180 wide. Copulatory bursa well developed. Penis cone-shaped (Fig. 4).

Female: Trunk 4.4 (4–5) mm long by 0.55 (0.4–0.7) mm wide (Fig. 1). Globular proboscis 210 (180–250) long by 180 (160–200) wide, armed with 11 rows of 3 hooks each, C I = 85 (75–85), R = 65 (60–70), C II = 45 (40–55), C III = 30 (25–45). Double-walled proboscis receptacle 300 (250–380) long by 110 (90–130) wide (Fig. 5). Lemnisci large and coiled. With single vaginal sphincter. Genital pore subterminal (Fig. 6).

Egg: Not available.

Holotype: Museum National d'Histoire Naturelle, Paris (MNHN). Hı 108.

Allotype (female): MNHN. Hı 107.

Paratype: MNHN. Hı 109.

Type host: Thermaceres andersoni Rosenblatt and Cohen, 1986 (Zoarcidae). MNHN-1985-400.

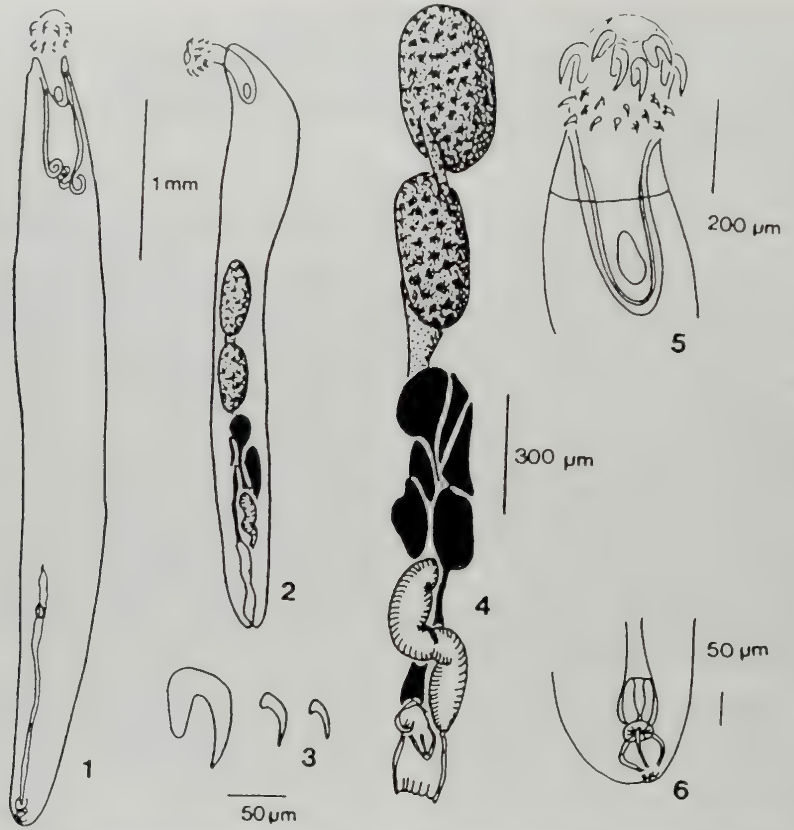
Site of infection: Small intestine.

Type locality: East Pacific Rise (12°48′N, 103°56′W) at 2,620 m depth. Pacific Ocean.

Etymology: Specific name *thermaceri* from the generic designation of the type host.

DISCUSSION

The genus *Hypoechinorhynchus* Yamaguti, 1939, presently includes 2 species: *H. alaeopis* Yamaguti, 1939, and *H. magellanicus* Szidat, 1950. The arrangement of the 40 proboscis hooks of *H. magellanicus* as described in the original description (Szidat, 1950) is 20 longitudinal rows; 5 rows of 2 hooks and 1 spine, 5 rows of 1 hook and 2 spines, and 10 rows of a single spine. It is more consistent with other species to view this arrangement as comprising 10 rows; 5 rows of 2 hooks and 2 spines and 5 with 1 hook and 3 spines. *Hypoechinorhynchus thermaceri* differs



FIGURES 1–6. *Hypoechinorhynchus thermaceri* n. sp. **1.** Female. **2.** Male. **3.** Proboscis hooks of a female. **4.** Reproductive system of a male. **5.** Proboscis of a female. **6.** Reproductive system of a female.

from *H. alaeopis* and *H. magellanicus* in both the number of longitudinal rows and the total number of hooks and spines (Table I). Further, the large rooted hooks of *H. thermaceri* are about the same size as those of *H. alaeopis* but are much smaller than those of *H. magellanicus.* The small hooks and the rootless spines of *H. thermaceri* are about the same size as those of *H. magellanicus* but are larger than those of *H. alaeopis* (Table I). *Hypoechinorhynchus alaeopis* is known to occur in Callionymidae from the subantarctic region of the Indian Ocean and in the western Pacific (Japan). *Hypoechinorhynchus magellanicus* occurs in Nototheniidae, also from the subantarctic region (Patagonia). *Hypoechinorhynchus thermaceri* is reported from the eastern Pacific (Central America) (Table I).

The ty[illegible] of *H. thermaceri* n. sp. is *Thermaceres a*[illegible] of the Zoarcidae. Zoarcid fish are already known to harbor acanthocephalans in their gut. *Zoarces viviparus* has been reported as a host of *Acanthocephalus lucii* (Muller, 1777) by Arro (1964); of *Pomphorhynchus laevis* Zoega *in* Muller, 1776, by Markowski (1933), Lundstrom (1942), Arro (1964), Willemse (1968), and Moller (1975); of *P. proteus* (=*P. laevis*) by Markowski (1938); of *Echinorhynchus gadi* Zoega *in* Muller, 1776, by Markowski (1938), Lundstrom (1942), Polyanski (1955), Arro (1964), Puidak (1965), and Moller (1975). In addition, *Zoarces viviparus* has been reported as the host of *E. salmonis* Muller, 1784, by Markowski (1938) and Lundstrom (1942), and of *Neoechinorhynchus rutili* (Muller, 1780) by Markowski (1938); Dollfus (1931) described *Echinorhynchus abyssicola* from *Pachycara obesa*; Paggi and Orecchia (1972) reported *Blennius pavo* as a host of *Golvanacanthus blennii*

TABLE I. *Comparison of characters of* Hypoechinorhynchus alaeopis *Yamaguti, 1939, and* H. magellanicus *Szidat, 1950, with* H. thermaceri *n. sp.*

Genus		*Hypoechinorhynchus* Yamaguti, 1939			
Species		*H. alaeopis* Yamaguti, 1939		*H. magellanicus* Szidat, 1950	*H. thermaceri* n. sp.
Hosts		*Alaeops plinthus* (= *Poecilopsetta plinthus*)[1] *Callionymus altivelis*[1] *Callionymus calauropomus*[2]		*Eleginops maclovinus*[3] *Parachaenichthys charcoti*[4] *Notothenia neglecta*[5] *Notothenia squamifrons*[6,7] *Notothenia rossi rossi*[7] *Notocheirus hubbsi*[8]	*Thermaceras andersoni*
Locality		Western Pacific (Japan)[1] Subantarctic Indian Ocean (Australia)[2]		Subantarctic (Argentina)[3–5, 8] Indian Ocean[6, 7]	Eastern Pacific (hydrothermal vents) (103°W, 13°N)
Authors		[1]Yamaguti (1939) [2]Johnston and Edmonds (1947)		[3]Szidat (1950) [4]Szidat (1965) [5]Szidat and Graefe (1967) [6]Parukhin and Zaitsev (1984) [7]Parukhin (1986) [8]Gosztonyi (1972)	
		(1)	(2)	(3)	
Male	Trunk length	2.50–3.30 mm	1.30–2.70 mm	1.50–1.80 mm	3.50 mm
	Trunk width	0.65–0.85 mm	0.45–0.70 mm	0.80 mm	0.45 mm
	Hooks	10 rows of 2 or 3 hooks each (total: 25) Anterior hooks with a root Posterior hooks rootless spines		10 rows of 4 hooks each (total: 40) Anterior hooks with a root Posterior hooks rootless spines	12 rows of 3 hooks each (total: 36) Anterior hooks with a root Posterior hooks rootless spines
	Length C I	69–80	71–101	120–150	85
	Length C II	27–33	30–36	50	50
	Length C III	20–27	21–32	40	40
	Ant. test. length, width	540–680 180–400	210–380 200–260	—	480–250
	Post. test. length, width	450–800 250–440	200–320 180–250	—	400–180
Female	Trunk length	2.70–8.00 mm	1.60–2.80 mm	3.00–5.00 mm	4.00–4.50 mm
	Trunk width	0.75–1.25 mm	0.50–0.91 mm	—	0.40–0.70 mm
	Hooks	10 rows of 2 or 3 hooks each		—	11 rows of 3 hooks each
	Length C I	69–96	74–110	—	—
	Length C II	36–38	38–42	—	—
	Length C III	27–32	28–35	—	—
	Egg length, width	57–60 16–18	50–54, 13–16	50–53, 20	Not available
Intermediate host		Unknown		*Exosphaeroma* (Isopoda)	Unknown

Paggi and Orecchia, 1972; and Van Maren (1979) found *P. laevis* in *Blennius fluviatilis*.

Among these fish hosts, only *P. obesa* is an abyssal species, having been caught at a depth of 4,785 m.

Campbell (1983) noted that few taxa of acanthocephalans are represented in abyssal fish because of their complex life cycle which includes an intermediate host and often 1 paratenic host. However, relative to this point, it is known that amphipods, decapods, gastropods, and pogonophorans occur at depths of approximately 2,600 m (Desbruyeres et al., 1982), hence an ecosystem exists at such sites. In fact, *Hypoechinorhynchus thermaceri* n. sp. is the second acanthocephalan to be found from an abyssal fish. It is notable that Rosenblatt and Cohen (1986) thought that "*Thermaceres* could be a derivative of a *Pachycara* fish" in which genus the first abyssal acanthocephalan was found.

Most of the fish observed to date at 13°N, 103°W seem to be *Thermaceres andersoni* (Fustec et al., 1987). Another species, *T. cerberus*, occurs off the coast of Mexico (21°N, 110°W) in the Galapagos Rift zone (Rosenblatt and Cohen, 1986); however, Geistdoerfer (1985) synonymized it with *T. andersoni*. Examination of the stomach of the paratype of *T. andersoni* revealed that it was empty, but examination of the stomach contents of specimens of *T. cerberus* revealed the presence of trochiform gastropods and lysionassid amphipods (Rosenblatt and Cohen, 1986). Consequently, the intermediate host of *H. thermaceri* n. sp. could be a crustacean of hydrothermal vents. In effect, Buron and Golvan (1986) showed that acanthocephalans from the superfamily Echinorhynchoidea have amphipods for intermediate hosts. One exception is *Exosphaeroma* (isopod), which is supposed to be the intermediate host of *Hypoechinorhynchus magellanicus* (Szidat, 1950). Without further evidence, this remains doubtful.

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RESEARCH NOTES

J. Parasit., 74(2), 1988, pp. 343–344

First Isolation of *Trypanosoma cruzi* from a Wild-caught *Triatoma sanguisuga* (LeConte) (Hemiptera: Triatominae) in Florida, U.S.A.

Charles B. Beard*, David G. Young*, and Jerry F. Butler,* and David A. Evans†, *University of Florida, Institute of Food and Agricultural Sciences, Entomology and Nematology Department, 3103 McCarty Hall, Gainesville, Florida 32611 and †Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, Keppel Street, Gower Street, London, United Kingdom WC1E 7HT.

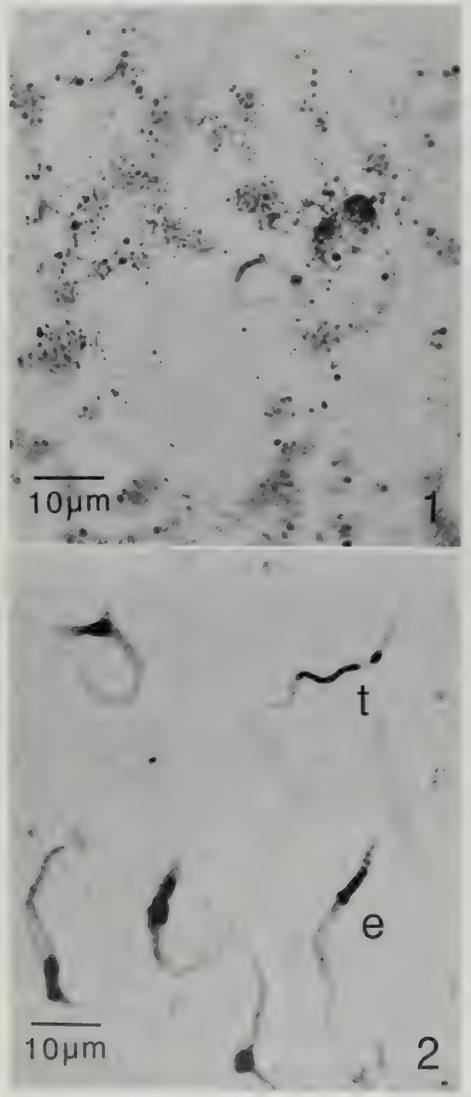
ABSTRACT: An adult female *Triatoma sanguisuga* was found to be naturally infected with *Trypanosoma cruzi*. This is the first report of a *T. cruzi* infection in this bug in Florida and suggests that a study of trypanosomiasis in reservoirs and vectors in north Florida is warranted.

Chagas' disease is an important cause of morbidity and mortality in the New World, affecting an estimated 10–20 million individuals (World Health Organization, 1985, Weekly Epidemiological Record **60:** 37–42). Both the causal agent, *Trypanosoma cruzi,* and *T. cruzi*-like organisms have been observed on numerous occasions in mammalian reservoirs and, less frequently, in triatomine bugs in the southern United States, but the disease is rarely diagnosed in man (Kagan et al., 1966, Revista de Biologia Tropical **14:** 55–73; Burkholder et al., 1980, Journal of Parasitology **66:** 305–311). To date, only 3 autochthonous human cases have been confirmed in the U.S.A. (Schiffler et al., 1984, Journal of the American Medical Association **251:** 2983–2984).

In Florida, *Triatoma sanguisuga* (LeConte) is the most important potential vector. The infection rate in this species varies according to locality (Olsen et al., 1964, Journal of Parasitology **50:** 599–603; Yeager, 1982, *In* Parasitic zoonoses, Vol. 1, L. Jacobs and P. Arambulo (eds)., CRC Press, Boca Raton, Florida, pp. 105–119). The only other indigenous triatomine species, *T. lecticularia* (Stål), is rarely encountered (Thurman et al., 1948, Florida Entomologist **31:** 58–62). Both species have been reported to be naturally infected with *T. cruzi* (Lent and Wygodzinsky, 1979, Bulletin of the American Museum of Natural History **163:** 127–520). Trypanosomes have been cultured from mammals in northern Florida (McKeever et al., 1958, Journal of Parasitology **44:** 583–587), but no naturally infected triatomines have been collected in that region (Irons and Butler, 1978, Florida Entomologist **61:** 31–33).

The infected bug reported here, an adult female *T. sanguisuga,* was collected at night from a screen door on a lighted porch of a Gainesville residence on 3 September 1986. The following day, we fed the bug on a restrained BALB/c mouse, and afterwards, examined the bug's feces in saline by phase-contrast microscopy. Trypomastigotes with large subterminal kinetoplasts were plentiful (Fig. 1). Another drop of feces from the bug was inoculated into a blood-agar slant tube overlaid with phosphate-buffered saline, pH 7.2, and supplemented with 1 drop each of penicillin–streptomycin and kanamycin sulfate antibiotic solutions. The culture was checked weekly until 10 October 1986 at which time epimastigotes and slender trypomastigotes were observed (Fig. 2). The parasites grew at 37 C in a mouse connective tissue cell line, NCTC clone 929, obtained from the American Type Culture Collection. Amastigotes were observed in fibroblast-like cells (Fig. 3) 1 wk following inoculation of parasites derived from the blood-agar slant tubes. One of us (D.A.E.) characterized the strain by isoenzyme profile as a member of the Zymodeme 1 group of *T. cruzi* (Miles, 1983, Transactions of the Royal Society of Tropical Medicine and Hygiene **77:** 5–23).

We have examined 99 specimens of *T. sanguisuga* from northern Florida since 1975, but this is the first bug found to be naturally infected with *T. cruzi.* Although most of our collections have been from sylvatic habitats, we also collected bugs inside a home near Lake Lochaloosa, south of Gainesville, where they had bitten the occupants on multiple occasions. Regardless of the apparent low infection rate in bugs, this con-



FIGURES 1, 2. Light micrographs of the Gainesville strain of *Trypanosoma cruzi*. **1.** Trypomastigote in feces of naturally infected *Triatoma sanguisuga*. **2.** Twenty-day culture forms, trypomastigote (t) and epimastigote (e).

FIGURE 3. Light micrograph of *Trypanosoma cruzi* amastigotes in a murine fibroblast cell line.

firmed isolation of *T. cruzi* suggests that a study of American trypanosomiasis in reservoirs and vectors in north Florida is warranted. This article is Florida Agricultural Experiment Station No 8442.

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A Natural Infection of *Fascioloides magna* in a Llama (*Lama glama*)

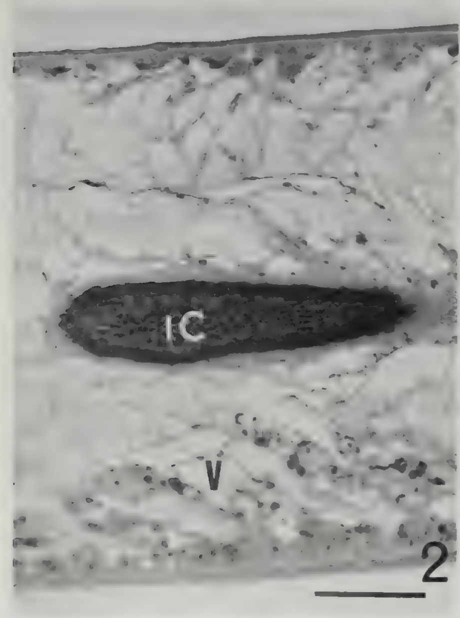
G. A. Conboy, T. D. O'Brien, and D. L. Stevens*, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108. *Present address: P.O. Box 1997, Vista, California 92083

ABSTRACT: A young, female llama (*Lama glama*) was euthanized following the onset of hindleg paresis and paralysis. Live trematodes, identified as *Fascioloides magna*, were identified from the liver of this animal. This represents the first report of *F. magna* in a llama.

A 1½-yr-old, female llama (*Lama glama*) was examined at the University of Minnesota Veterinary Teaching Hospital, because of hindleg paresis. The paresis later progressed to paralysis and the llama was euthanized. During necropsy, lesions unrelated to the central nervous system disease were seen in the liver. The liver parenchyma contained multiple necrotic tracts, each surrounded by a thin capsule of gray–white connective tissue. Microscopically the tracts consisted of a central area of necrosis and hemorrhage, with neutrophils, macrophages, lymphocytes, and plasma cells present. Some of the tracts contained live trematodes. Five flukes ranging in length from 8 to 20 mm ($\bar{x}$ = 13.6 mm, SD = 5.5 mm) were recovered from the liver.

The flukes were fixed in 10% buffered formalin and the 4 smallest flukes were stained in Semichon's acetic carmine. The largest fluke was cut in cross section, embedded in paraffin, and a 5-μm section was mounted on a slide and stained with hematoxylin and eosin. The anterior end of the flukes was rounded in shape and the intestinal ceca were highly branched (Fig. 1). Genital primordia consisting of a cirrus, uterus, ootype, a branched ovary, and 2 branched testes were visible in the whole-mounted flukes. The uterus and the ovary were located anterior to the ootype. The testes were posterior to the ootype. There were no ova present in any of the flukes. In the cross section, the vitelline glands were located almost entirely ventral to the intestinal ceca (Fig. 2). The anatomy of the flukes appeared identical to that as described for *Fascioloides magna* (Bassi, 1875), the large American liver fluke (Stiles, 1894, Journal of Comparative Medicine and Veterinary Archives **15:** 161–178, 225–243, 299–313, 407–417, 457–462; Swales, 1935, Canadian Journal of Research **12:** 177–215). The flukes were similar in size, and identical in morphology to immature *F. magna* recovered in this laboratory from experimentally infected sheep and guinea pigs 3–4 mo postinfection.

Fascioloides magna infection in llamas has not been reported previously. This llama was born and raised on a hobby farm located in the northern half of the state of Minnesota. The area has a large population of white-tailed deer (*Odocoileus virginianus*). White-tailed deer are a natural definitive host of *F. magna*. Adult flukes reside in cysts in the liver parenchyma, which open to the biliary system. Flukes mature to patency at about 7 mo postinfection, the ova passing through the bile ducts into the small intestine and out in the feces. Various lymnaeid snails serve as intermediate hosts. The deer are infected by eating metacercaria encysted on vegetation. *Fascioloides magna* does not seem to be very pathogenic to white-tailed deer (Foreyt and Todd, 1976, Journal of Parasitology **62:** 26–32). Sheep, cattle, horses (Swales, 1935, loc. cit.), goats (Foreyt and Leathers, 1980, American Journal of Veterinary Research **41:** 883–884), pigs (Migaki et al., 1971, American Journal of Veterinary Research **32:** 1417–1421), and various other animals (Swales, 1935, loc. cit.) can serve as aberrant hosts. In cattle *F. magna* is totally encapsulated in cysts in the liver which do not open to the biliary system (Swales, 1935, loc. cit.). Rarely, flukes are also found in the lungs of infected cattle (Foreyt and Todd, 1976, loc. cit.). Death loss in cattle due to *F. magna* infection has been thought to be rare. Most infected cattle, as with white-tailed deer, show little or no clinical sign of infection. In sheep, however, infection with *F. magna* is usually fatal; the animals die within 6 mo of infection. Infection with relatively few flukes has been found to kill a sheep. Flukes migrate freely through the liver, lungs, and other tissues (Foreyt and Todd, 1976, loc. cit.; Stromberg et al., 1985,



FIGURES 1, 2. *Fascioloides magna*. **1.** A photograph of a stained, immature *Fascioloides magna*, 1 of 5 recovered from the liver of a llama. Bar = 1 mm. **2.** A photomicrograph of one of the flukes in cross section. The vitelline follicles (V) were located entirely ventral to the intestinal ceca (IC). Bar = 100 μm.

American Journal of Veterinary Research **46:** 1637–1641). Sheep seem unable to encapsulate migrating flukes. Because of the capsule formation seen in the liver of this llama in response to fluke infection, it is likely that *F. magna* infection in llamas is similar to that in cattle. It is probable that *F. magna* is not a serious pathogen in llamas imported into endemic areas.

J. Parasit., 74(2), 1988, pp. 347–349

Distribution of *Spiculopteragia pursglovei* and *S. odocoilei* (Nematoda: Trichostrongyloidea) from White-tailed Deer (*Odocoileus virginianus*) in the Southeastern United States

Dominic A. Strohlein*, Church B. Crow†, and William R. Davidson†, *U.S. Department of Agriculture, Animal Parasitology Institute, BARC-East, Beltsville, Maryland 20705 and †Southeastern Cooperative Wildlife Disease Study, Department of Parasitology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602

ABSTRACT: The distribution of *Spiculopteragia pursglovei* (=*Apteragia pursglovei*) and *S. odocoilei* (=*A. odocoilei*) in 12 southeastern states was determined after examining the abomasal contents of 1,369 white-tailed deer over an 8-yr period. *Spiculopteragia odocoilei* was encountered with much greater frequency than *S. pursglovei* except in some areas along the Mississippi River drainage and the coasts of North Carolina and South Carolina. In instances where both parasites were found in a population, one usually expressed a dominance in both prevalence and intensity. These findings are in agreement with those of an earlier study conducted in the southeastern United States.

In describing *Spiculopteragia pursglovei* (=*Apteragia pursglovei*) Davidson and Prestwood (1979) made note of its patchy distribution in the southeastern United States in relation to the frequency with which *S. odocoilei* (=*A. odocoilei*) had been encountered. Among the 152 counties in 13 southeastern states from which white-tailed deer were taken, *S. odocoilei* was identified as the dominant species in all counties except those found along large river drainages and coastal areas where *S. pursglovei* was more abundant (Fig. 1). Of the 824 deer examined in their study, 76.5% had only *S. odocoilei*, 13.8% had only *S. pursglovei*, 5.0% had both species, and 4.7% had neither.

Since that investigation, abomasa from an additional 1,369 white-tailed deer from 140 counties in 12 southeastern states have been examined. Identification of species of *Spiculopteragia* was based on the size, conformation, and degree of sclerotization of the spicules (Davidson and Prestwood, 1979). In the present study classification of *Spiculopteragia* was done in accordance with the scheme of Durette-Desset (1983) who record *Apteragia* as a synonym of *Spiculopteragia*. The distribution of *Spiculopteragia* from this latter group of deer is shown in Figure 2. There is general agreement between our findings and those of Davidson and Prestwood (1979). *Spiculopteragia odocoilei* was the dominant species in most areas, with exceptions occurring along the Mississippi River drainage and the coastal areas of North Carolina and South Carolina. Other inland foci occurred but in these instances *S. pursglovei* was found infrequently and with low intensity.

In the present study, the states with the highest prevalence of *S. pursglovei* were Louisiana (53%), Arkansas (33.7%), North Carolina (28.5%), and South Carolina (26.4%). *Spiculopteragia odocoilei* was found in at least 70% of the deer in all states except North Carolina (53.3%), Arkansas (37.6%), and Louisiana (32.3%). Of all the white-tailed deer examined, 7.1% had both species of *Spiculopteragia* and 0.6% had neither. In cases of co-occurrence, one species usually dominated over the other in both prevalence and intensity. This remained true from year to year in areas where consecutive samples were taken. In the present study, mixed infections were found more frequently along the southern reaches of the Mississippi River than previously reported (Davidson and Prestwood, 1979).

Changes in geographic distribution of deer populations and translocation of deer during restocking programs are some possible explanations for the distribution of the 2 species. As noted by Davidson and Prestwood (1979), the past practice of translocating deer in the Southeast can confound interpretation of these data. However, *S. pursglovei* appears to remain conservative in its distribution in the southeastern United States. This suggests that *S. pursglovei* may be restricted to certain habitat types necessary for the successful maintenance of its life cycle and/or that competition between the 2

species is expressed as dominance of one or the other within a given deer population.

This study was supported by an appropriation from the Congress of the United States. Funds were administered and research coordinated under the Federal Aid in Wildlife Restoration Act (50 Stat. 917) and through Contract Numbers 14-16-0009-78-023, 14-16-0009-80-008, 14-16-0009-81-014, 14-16-0009-82-500, 14-16-0004-83-004, 14-16-0004-84-005, 14-16-0004-85-007, and 14-16-0004-86-910, Fish and Wildlife Service, U.S. Department of the Interior.

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DURETTE-DESSET, M. C. 1983. No. 10 keys to the genera of the superfamily Trichostrongyloidea. *In* CIH keys to the nematode parasites of vertebrates, R. C. Anderson and A. G. Chabaud (eds.). Commonwealth Agricultural Bureaux, Farnham Royal, Buckinghamshire, England, 86 p.

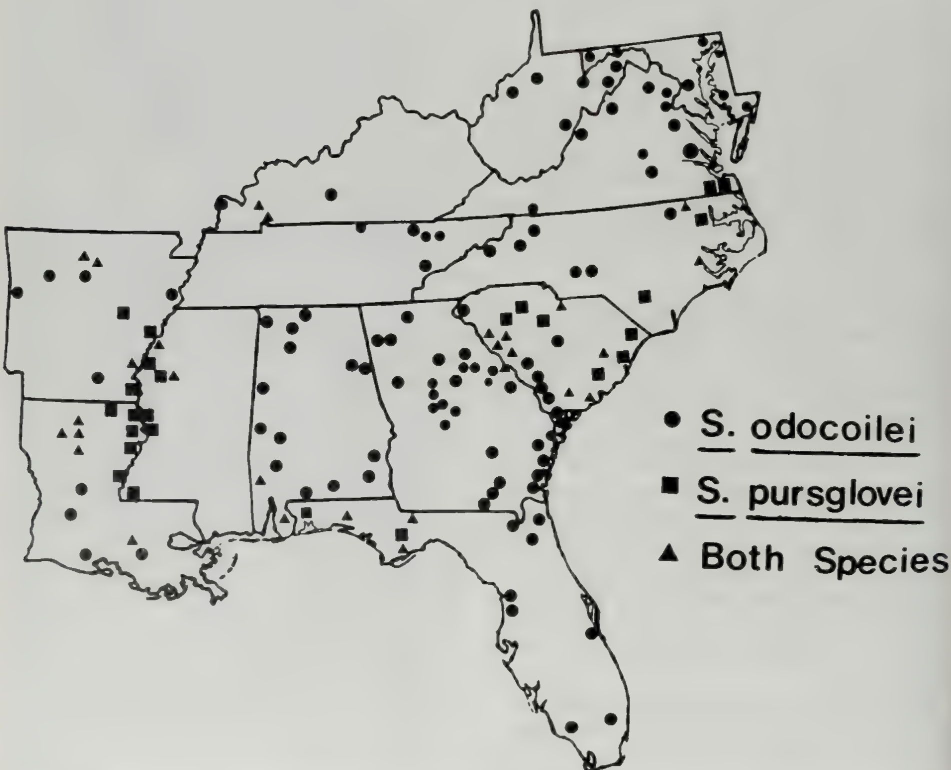

FIGURE 1. Distribution of *S. odocoilei* and *S. pursglovei* in the southeastern United States (1962–1978) (after Davidson and Prestwood, 1979).

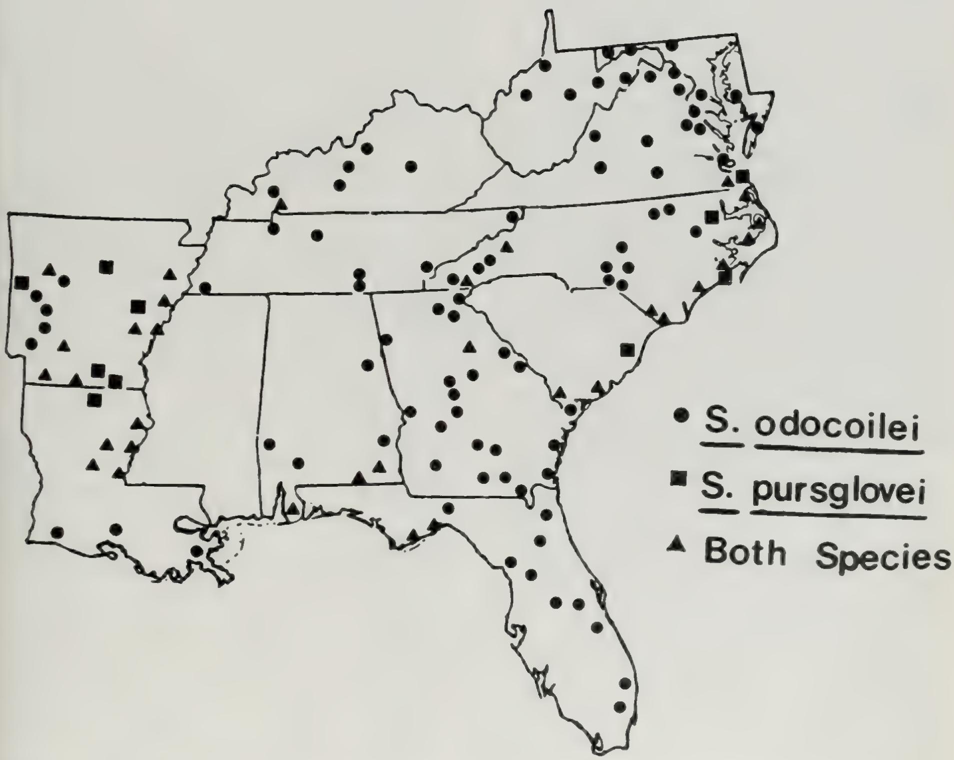

FIGURE 2. Distribution of *S. odocoilei* and *S. pursglovei* in the southeastern United States (1978–1985).

J. Parasit., 74(2), 1988, pp. 350–351

BOOK REVIEW . . .

Geschichte der Helminthologie im deutschsprachigen Raum, Karl Enigk, Dr. habil. Professor emeritus für Parasitologie der Tierärztlichen Hochschule Hannover. Mit einem "Geleitwort" von Johannes Eckert, Prof. Dr. Leiter des Instituts für Parasitologie der Universität Zürich. Gustav Fischer Verlag, Stuttgart, New York 1986.

The volume opens with a "Vorwort" by the author, followed by an eight-page "Begleitwort," featuring a photograph and biographical sketch of the author and 54 titles, selected from his publications.

In the "Vorwort," the author outlined the purpose and design of the book. The purpose is clearly stated in the title: to record the story of the parasitic worms that infest man and other vertebrate animals as it developed and was uncovered and unfolded by investigators and authors in the German-speaking area. There is a Table of Contents, arranged chronologically, but no figures, no bibliography, and no index. The last section of the book is a "Namensregister" of eight pages, and some 1,000 names of authors cited in the text. The literature list is enormous and for bibliographic details, the reader is referred to the Index-Catalogue of Medical and Veterinary Zoology, published by the U.S. Department of Agriculture, Washington, D.C. For important contributions, not included in the Index-Catalogue, there is a "Schrifttum" of six pages with full bibliographic information. The text is embellished by 58 photographs of German authors, with brief biographical data, and in addition there is a section (pp. 273–339) with supplementary biographical information. The text is restricted to German authors, but contributions by others are considered when they affect the situation in Deutschland.

The story of the parasitic worms, as retold by Prof. Enigk, is detailed and remarkedly complete. It is a notable achievement to celebrate on his 80th birthday.

It begins with a reference to Hippocrates (460–370 B.C.); he and his students knew these worms and called them Helminthen. They were the only known causes of illness and there was wide belief that they arose by spontaneous generation in decomposing organic material. With the decline of Greek culture, the study of natural history entered the dark ages, and for more than a thousand years there were few advances. The 17th century witnessed a rise in interest in human health and animal welfare. Throughout Europe, physicians and veterinarians became alert, looking for causes of disease, including infections by parasitic worms. Francisco Redi (1620–1698), physician and professor at the University of Pisa, studied stages in the development of worms found in the bodies of various vertebrates and in a publication (1684) described 108 species of parasitic worms. Enigk designated him "der Begründer der Parasitologie." Marcello Malpighi (1628–1694) introduced the microscope into biology and medicine. Dissections of many animals yielded larval stages of parasitic worms. He reported *Cysticercus cellulosae* in pork and opposed spontaneous generation. Antonio van Leeuwenhoek (1632–1725), artisan of Delft, built a microscope with lenses that magnified 270 times. He studied small aquatic organisms and stages of developing worms. Jan Swammerdam (1657–1680), physician and naturalist of Amsterdam and Leiden, made microscopic observations of all kinds of invertebrates, with results that were recorded in his "Bibel der Natur," with editions and translations. He described cercariae from snails but failed to recognize their significance. Nicolas Andry (1658–1742) reviewed medical aspects of human helminthology in a "Traité de la génération des vers dans le corps de l'homme, de la nature et des espèces de cette maladie, de ses effets, de ses signes, de ses prognostics." 470 p., 5 pl., 1700, Paris. German translation, "Die Helminthen des Menschen, 1716."

Until 1850, advances in helminthology were based on descriptive reports. Ideas of interrelations and classification rested on morphological data. In 1851, Küchenmeister (1821–1898) fed 40 *Cysticercus pisiformis* to young foxes and killed them at intervals of 39 hr, 8, 15, and 22 days. He recovered 35 specimens representing developmental stages of the cestode. He then fed *Cysticercus tenuicollis* and *Coenurus cerebralis* to dogs and *Cysticercus fasciolaris* to cats with the development and recovery of sexually mature cestodes. Enigk declared, "Damit hatte Küchenmeister zum ersten Male die vollständige Entwicklung eines Bandwurmes experimentell bewiesen."

The nematodes constitute the largest and in some respects the most significant group of the parasitic worms. Enigk noted (p. 136) that with the exception of *Trichinella spiralis,* the biology of the round worms was totally unknown until 1861. It was generally believed that all nematodes had alternation of generations. In 1861, there was an outbreak of trichinosis in the area of Plauen and a girl died. Zenker (1815–1898) studied the musculature and found many larvae. He fed the material to dogs with negative results. He gave material to Leuckart and Virchow. Leuckart fed the tissue to mice and 7 days later recovered sexually mature worms. The females were producing larvae, not eggs. The larvae were being distributed by the vascular system and were encysting in striated muscle. This experiment showed that *T. spiralis* is a one-host species.

Swammerdam had described cercariae; von Siebold and other observers had noted similarities between cercariae and trematodes. Attempts at life cycles were reported, but the experiments were uncontrolled, incomplete, or otherwise delinquent; specific identity was doubtful, and the first credible report was that of *Fasciola hepatica,* done independently and simultaneously by Leuckart in Germany and Thomas in England, in 1882. The trematodes are unique in species that have multiple, repeated, asexual generations. Donges in experiments (1963–1970) transplanted rediae of *Isthmiophora melis* to uninfected snails and in 4 yr obtained 40 generations of rediae without a sexual generation. Repeated infections by miracidia produced no immunity.

With the discovery of complicated life cycles, alternations of generations, and successive generations in different hosts, eyes and minds were opened and former restrictions were relaxed. Enigk declared (p. 144), "Durch die Klärung der Lebenskreise konnte die Taxonomische wie auch die phylogenetische Stellung dieser Helminthen sicherer gemacht werden."

Section 8 of the Geschichte is entitled "Helminthologen von 1861–1900." This was the golden age of helminthology in Germany. There were many investigators and many areas of research. The leader of the German contingent was K. G. F. Rudolph Leuckart (1822–1898). His recognition was international. A student of the invertebrates, his numerous publications, especially his successes on the life cycles of parasitic worms, brought his doctorate in 1845 and his call to Giessen in 1850. Here, his achievements were continued, with the call to Leipzig in 1869. The Leipzig school became famous, with students from European countries and North America. Leuckart was an active member of the University Administration, a gifted lecturer, an eminent author, director of the doctoral research of 137 students, and the recipient of many honors. The University celebrated his 70th birthday with a Festschrift in which 148 students registered their gratitude. A marble bust was dedicated by students in 1895. At the Second International Congress of Parasitology, held in Munich, the Deutsche Gesellschaft für Parasitologie celebrated his 150th birthday.

Enigk noted the reduction in research at the end of the century. The field of helminthology was extended and divided into areas of special interest to physicians and veterinarians. Moreover, the international situation was deteriorating and in 1914, the First World War interrupted research. The European countries were exhausted, impoverished; inflation was rampant, and living costs were multiplied. Leuckart's ablest student, Arthur Loose (1861–1923), world famous helminthologist, was dismissed from his position in Egypt and, without his personal effects, was returned to Germany. Here, without funds or stipend, he starved to death. The greatest loss from the war was the intelligence that perished in the casualty lists.

H. W. Stunkard, American Museum of Natural History, New York, New York 10024.

J. Parasit., 74(2), 1988, p. 352

BOOK REVIEW . . .

The Rothschild Collection of Fleas. The Ceratophyllidae: Key to the Genera and Host Relationships, by Robert Traub, Miriam Rothschild, and John F. Haddow. Privately published by Miriam Rothschild and Robert Traub. Distribution by Academic Press Inc., 24–28 Oval Road, London NW1 7DX. 1983. xv + 288 p. + 271 plates and maps. U.S. $93.00.

The long-awaited volume on the fleas of the family Ceratophyllidae has arrived, although it is not in the format of the well-known *Illustrated Catalogue of the Rothschild Collection of Fleas.* In the preface we are told that the ceratophyllid fleas comprise about 20% of all known fleas. The vast number of species has necessitated a change of format. A key to the genera and subgenera is given, amply illustrated with photographs and line drawings, but unlike the *Illustrated Catalogue* no detailed account of the individual species is included. The color and imprinting on the binding are both very similar to the six-volume *Illustrated Catalogue,* but physically this volume is smaller.

The opening chapter, Key to the Genera and Subgenera of Ceratophyllidae, is the very backbone of the book. This chapter was contributed by F. G. A. M. Smit, who declined the invitation to be a coauthor of the volume. Sixty-five genera and subgenera are considered, among which are seven new genera and eight new subgenera. The family characteristics and those of the new genera and subgenera are carefully described. In the key each diagnostic character is accompanied by a reference to a figure which illustrates the character. The 205 line drawings and 90 plates of photographs are exceptionally good and illustrate the taxonomically important features of the ceratophyllids. The illustrations and the identification key work so well together that even a nonspecialist can use the key with some effort. Ninety plates of photographs further illustrate each genus and subgenus. The photographs are retouched to enhance contrast and are exceptionally good.

A map and a five-page listing of regions and subregions are used to explain the zoogeographical classification that is used. New subdivisions at the subregional level are given the new term sectors.

The chapter on the distribution of ceratophyllid fleas and notes on their hosts does an excellent job of condensing much information into relatively little space. Each genus is discussed briefly and a map of its worldwide distribution is depicted. Every subgenus and species is listed along with data on hosts, geographic distribution, and ecological notes (often just elevations of occurrence). There is also a reference to a distribution map for each species. On the maps shading is used to illustrate the host distribution and a variety of symbols indicate flea collection sites. Some of the shading is a little subtle and hard to see, but in general it is accurate and informative. The only error that I noted was on map 28, where the shaded area is supposed to show the distribution of *Tachycineta bicolor,* the tree swallow, but instead shows the distribution of *Tachycineta thallassina.*

The chapter on The Hosts of the Ceratophyllid Fleas discusses the numerical data available on ceratophyllids on different host orders, host families, etc., of birds and mammals. A chapter entitled Evolution of the Ceratophyllidae discusses the coevolution of host and parasite and places the origin of the ceratophyllid fleas at the Oligocene (38–40 million years ago) originating in the New World along with the sciurid rodents.

The 26-page chapter Medical Importance of the Ceratophyllidae has a good bibliography and brief account of the species of ceratophyllids that play a role in transmission of plague, typhus, tularemia, etc.

Five appendices treat in tabular form the following: a list of the 25 species described since 1975; classification of hosts (bird and mammal orders and families) and number of genera and species of ceratophyllid fleas they carry; a host list with major ceratophyllids; maps showing the distribution of the British Ceratophyllidae; and a list of taxa with current and old designations. This is followed by an Index that is very complete, accurate, and useful.

This volume will be indispensable to serious flea researchers for the identification key and because 15 new taxa are described. It is highly recommended also for other entomologists in general, and mammalogists, medical entomologists, zoogeographers, and evolutionary biologists.

Ralph P. Eckerlin, Natural Sciences Division, Northern Virginia Community College, Annandale, Virginia 22003.

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Department of Zoology
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ANALYSIS OF GLUTATHIONE-ENHANCED DIFFERENTIATION BY MICROFILARIAE OF *ONCHOCERCA LIENALIS* (FILARIOIDEA: ONCHOCERCIDAE) *IN VITRO*

R. J. Pollack*, J. B. Lok, and J. J. Donnelly†

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, Pennsylvania 19104

ABSTRACT: Reduced glutathione (GSH), but not its oxidized form (GSSG), stimulated development of *Onchocerca lienalis* microfilariae to the late first-larval stage *in vitro*. The degree and frequency of development was dose-related with a peak of activity at 15 mM, a concentration that is similar to known intracellular levels of GSH. To determine the mode(s) of action of this multifunctional compound, other reducing agents (L-cysteine, dithiothreitol), cysteine delivery agents (N-acetyl-L-cysteine, L-thiazolidine-4-carboxylic acid, L-2-oxothiazolidine-4-carboxylic acid), cysteine analogues (S-methyl-L-cysteine, D-glucose-L-cysteine, cysteine ethyl ester), free-component amino acids of GSH (glutamic acid, cysteine, and glycine), a specific metabolic inhibitor of γ-glutamyl synthetase (buthionine sulfoximine), and an inhibitor of γ-glutamyl transpeptidase (γ-glutamyl glutamic acid) were also tested at concentrations of 0.01–50 mM in this system. N-acetyl-L-cysteine at 1–5 mM and D-glucose-L-cysteine at 2.5–10 mM significantly enhanced development. In contrast to those worms maintained in GSH-supplemented medium, microfilariae exposed to GSH for only the first 24 hr showed no enhancement by day 7 in culture. Neither buthionine sulfoximine nor γ-glutamyl glutamic acid at 0.01–35 mM inhibited the effects of 15 mM GSH or 1 mM N-acetyl-L-cysteine. Results indicate that GSH or other cysteine analogues possessing a free sulfhydryl group must be present in the extranematodal environment to support microfilarial differentiation *in vitro*.

Methods for the *in vitro* cultivation of invertebrate-phase larvae of *Onchocerca* spp. are needed for research on their metabolism and for production of third-stage larvae for immunological and chemotherapeutic studies. Currently, production of infective and preinfective larvae is dependent upon the use of field- or laboratory-infected flies (Lok et al., 1983). Thorough reviews of attempts to culture filarial worms and discussions of physicochemical requirements were presented by Weinstein (1970, 1986). Recently, Schiller et al. (1979), using a biphasic culture medium, reported the development of a small number (1% or less) of *O. volvulus* microfilariae to stages equivalent to, and beyond, those normally occurring in the simuliid vector. Pudney and Varma (1980) reported development of *O. volvulus* and *O. gutturosa* microfilariae to the first-stage sausage forms in cultures containing vertebrate sera and cells of the tick *Rhipicephalus appendiculatus*. Devaney and Howells (1983) reported that up to 30% of *O. lienalis* microfilariae developed to the intermediate stage in Medium ML-15, which consisted of Leibovitz-15 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 10% tryptose-phosphate broth (TPB). Supplementation of ML-15 with 5 or 10 mM of glutathione or L-cysteine led to further enhancement of development with a maximum of 20% of the microfilariae reaching the full sausage form, i.e., the late first-stage larva. No development occurred when either the reducing agents dithiothreitol or L-ascorbic acid were substituted for glutathione or L-cysteine.

Glutathione (L-γ-glutamyl-L-cysteinylglycine), the most prevalent intracellular thiol, is known to function directly or indirectly in catalysis, metabolism, transport, and reductive processes and in protection of cells by destruction of free radicals, reactive oxygen intermediates, and other toxic compounds of endogenous and exogenous origin, and as a storage and transport form of cysteine (Meister et al., 1986). Selective modification of the transport or metabolism of glutathione is possible through the use of glutathione or cysteine delivery agents, or by inhibition of the enzymes of the γ-glutamyl cycle (Meister and Anderson, 1983; Anderson and Meister, 1986).

Among the parasitic helminths, the presence of glutathione or the enzymes glutathione S-transferase and γ-glutamyl transpeptidase have been confirmed in *Schistosoma mansoni* (Bue-

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* To whom reprint requests should be addressed.

† Department of Ophthalmology, Scheie Eye Institute, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104.

ding et al., 1982), *Echinococcus granulosus* (Morello et al., 1982), *Moniezia expansa* (Douch and Buchanan, 1978), *Haemonchus contortus* (Kawalek et al., 1984), *Ascaris suum* (Dass and Donahue, 1986), adult *Dirofilaria immitis* and *Brugia pahangi* (Jaffe and Lambert, 1986), and adult *Litomosoides carinii* (Bhargava et al., 1983).

The present investigations were initiated in order to ascertain the mode of action of glutathione and other reducing agents and thiols with respect to microfilarial development *in vitro*. Reduced glutathione, N-acetyl-L-cysteine, and D-glucose-L-cysteine enhanced the degree and frequency of development of *O. lienalis* microfilariae in dose-dependent relationships, whereas other cysteine analogues and thiols had no effect or inhibited development. Inhibitors of γ-glutamyl transpeptidase and γ-glutamylcysteine synthetase did not affect the action of GSH or N-acetyl-L-cysteine, suggesting that these specific transport and degradation mechanisms for GSH were not significantly involved in the effect of GSH on microfilarial development *in vitro*.

MATERIALS AND METHODS

Parasites

Microfilariae of *O. lienalis* were isolated from umbilical skin samples obtained from cattle at slaughter. Standard techniques for harvest (Lok et al., 1980) and cryopreservation (Ham et al., 1981) of microfilariae were followed. Each batch of microfilariae acquired was tested for viability by laboratory infection in laboratory-reared black flies, *Simulium vittatum* and *S. pictipes* (Lok et al., 1983). Just prior to inoculation into culture, fully motile fresh or cryopreserved microfilariae were purified by migration out of an agarose pad (Greene and Schiller, 1979). The agarose pad (0.8% low-gelling temperature agarose, Sigma Chemical Co.) contained and was overlaid with liquid L-15 (GIBCO) supplemented with 10% FBS, 100 μg/ml gentamicin sulfate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml chloramphenicol sodium succinate. After 1–2 hr incubation at 37 C in air, microfilariae in the liquid phase were centrifuged (50 *g* for 10 min) and resuspended in antibiotic-free L-15 medium.

Compound formulation

Aqueous stock solutions (0.1–1 M) of the test compounds were prepared daily, the pH adjusted to 7.0 with HCl, NaOH, or NH_4OH, filter-sterilized, and held at 4 C (4 and 25 C for L-cysteine) until final dilutions were made. Test compounds acquired from Sigma consisted of: glutathione, reduced (GSH); glutathione, oxidized (GSSG); L-cysteine (free base) (CYS); N-acetyl-L-cysteine (NALC); S-methyl-L-cysteine (SMLC); D-glucose-L-cysteine (DGLC); DL-dithiothreitol (DTT); L-thiazolidine-4-carboxylic acid (THIO); L-glutamic acid (GLU); L-glutamine (GLN); L-glycine (GLY); γ-L-glutamyl-L-glutamic acid (γGG); sucrose; trehalose; α-D(+)-glucose; DL-buthionine-(S,R)-sulfoximine (BSO). L-2-oxothiazolidine-4-carboxylic acid (OTC) was obtained from Aldrich Chemical Co. Tryptose-phosphate broth (TPB) and cysteine ethyl ester (CEE) were acquired from GIBCO. Powdered L-15 medium was obtained from both Sigma and GIBCO. The free sulfhydryl status of test compounds in aqueous solution was ascertained by the use of 5,5′-dithiobis (2-nitrobenzoic acid) in procedures reviewed by Jocelyn (1987).

Culture techniques and evaluation

Cultures were carried out in stationary 48-well plates. Each well contained 50–100 microfilariae in 0.5 ml medium. Culture medium was antibiotic-free L-15 (pH 7.6) supplemented with 10% FBS, and cultures were incubated at 27 C, at 95% relative humidity in an atmosphere of air. After 7 days in culture, during which no contamination was observed, all worms were examined at 100× magnification with an inverted microscope and each categorized as to morphological form and relative motility. Developmental categories, based on those of Devaney and Howells (1983), were: microfilaria, intermediate stage, early sausage stage, and full sausage stage and are reported herein as MF, S-1, S-2, and S-3, respectively. Note that these are all sequential developmental forms of the first-stage larva.

Experimental design

Microfilariae were inoculated into medium containing concentrations of test substances ranging from 0.01 to 50 mM. Development and motility of the worms in each experimental group were assessed with respect to those in control cultures. Experiments were performed as double-blind procedures. Triplicate cultures were carried out at each concentration and experiments were repeated at least 3 times. Percentage data were subjected to arcsine transformation prior to calculation of means. Mean angular values representing development to the S-3 form at all tested concentrations of reagents were used in calculating 1-way analysis of variance (ANOVA). Retransformed percentage data over the active range (concentrations of the reagent promoting development to the S-3 form) were employed for computer generation of second-order polynomial equations to provide a model for dose–response relationships. Histograms were constructed to indicate degree of microfilarial development at different reagent concentrations. As mean angular values were retransformed to percentage data, the sum of means may not total 100%.

RESULTS

Qualitative study

To qualitatively confirm the results reported by Devaney and Howells (1983), microfilariae were inoculated into wells of unsupplemented L-15 (control), and in L-15 supplemented with 10 mM GSH, 10% each of FBS and TPB, and combination of 10 mM GSH and 10% each of FBS and TPB. Addition of either 10 mM GSH or 10% each of FBS and TPB to L-15 supported development of over 80% of the microfilariae to

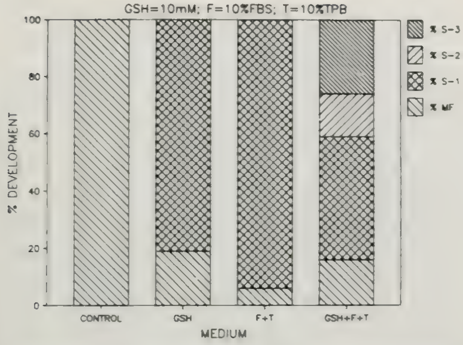

FIGURE 1. Development of *O. lienalis* microfilariae to sequential forms (S-1, S-2, S-3) of first-stage larvae in unsupplemented L-15 medium (control) or in L-15 supplemented with 10 mM GSH, 10% each of fetal bovine serum (FBS) and tryptose-phosphate broth (TBP), or a combination of these treatments. Cultures were incubated for 7 days at 27 C in an atmosphere of air. Histograms are based on percentage data, retransformed from mean angular values of observed proportions from 3 experiments, each with 3 replicates per treatment and 50–100 microfilariae per replicate.

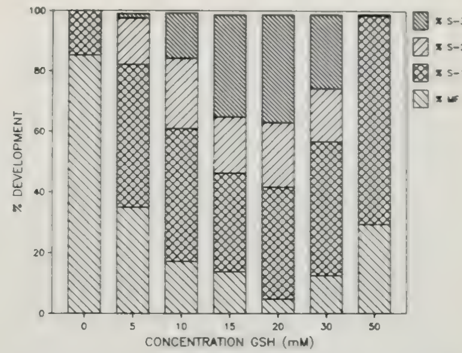

FIGURE 2. Developmental profile of microfilariae cultured in L-15 medium containing 10% each of FBS and TPB and further supplemented with 0–50 mM GSH. Values were derived from 3 experiments.

the S-1 form (Fig. 1). The effects of these combined additives were synergistic, with development occurring beyond the S-1 form to the S-2 and S-3 forms.

GSH quantitative study I

To determine whether the effects of GSH were dose-related, GSH was tested at final concentrations of 0, 5, 10, 15, 20, 30, and 50 mM. All wells contained L-15 supplemented with 10% each of FBS and TPB. Results of exposure to varying concentrations of GSH in this system indicated a dose-related response, with a peak in the degree and frequency of development at 15–20 mM GSH (Fig. 2). Nearly 40% of the microfilariae developed to the S-3 form at these concentrations.

GSH quantitative study II

To determine whether TPB, a major undefined medium supplement, was a major influence in the developmental response, TPB was excluded from the medium in this test. Although removal of the TPB from the culture medium reduced the mean frequency of microfilarial development (Fig. 3), the dose–response profile in this study was similar to that obtained in the GSH quantitative study I. In order to account for variations in development due to worm batch variability, such GSH controls (0–30 mM) were run concomitantly against all test substances. Results were used only when microfilarial development in GSH control cultures paralleled the profile obtained in this trial.

Osmolality

Osmolality-matched media containing either glucose, sucrose, or trehalose in place of GSH produced no developmental enhancement at concentrations of 0.5–50 mM (data not shown).

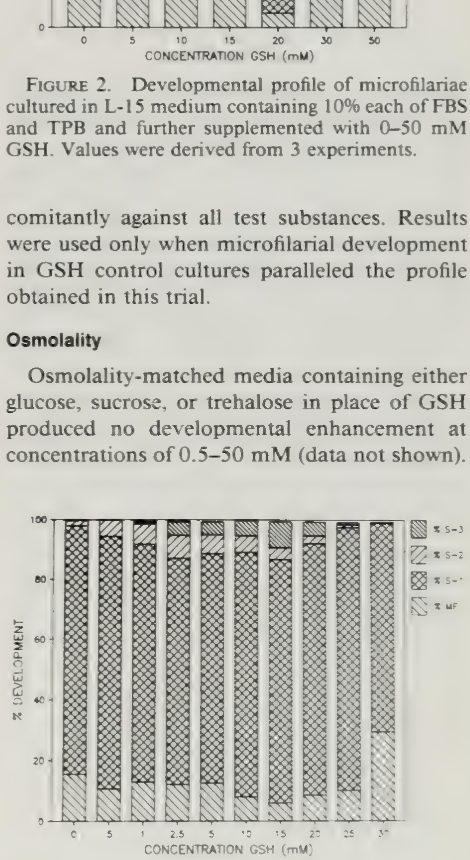

FIGURE 3. Developmental profile of microfilariae cultured in L-15 medium containing 10% FBS and supplemented with 0–30 mM GSH. Values were derived from 5 experiments. Mean angular values representing development to the S-3 form at all tested concentrations of GSH were used in calculating 1-way analysis of variance ($F = 8.6031$, $df1 = 9$, $df2 = 33$, $P < 0.001$). Retransformed percentage data over the range of GSH that promoted development to the S-3 form (1–30 mM) were employed for computer generation of the second-order polynomial equation of $Y = 0.060 + 1.165X - 0.39X^2$ (R-value = 0.94).

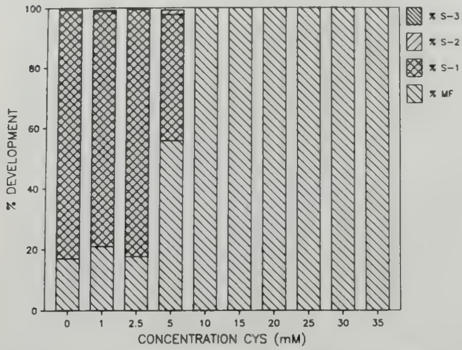

FIGURE 4. Developmental profile of microfilariae cultured in L-15 medium containing 10% FBS and supplemented with 0–35 mM cysteine. Values were derived from 3 experiments.

Reducing agents

To confirm results reported by Devaney and Howells (1983), development of *O. lienalis* microfilariae was examined in the presence of CYS and DTT at 0–35 mM and compared to GSH controls at these concentrations. At concentrations of 5 mM or less, CYS (Fig. 4) and DTT (data not shown) either had no effect or inhibited development with respect to CYS- and DTT-free controls. At higher levels, these compounds inhibited motility as well as development and tended to precipitate from solution.

GSH vs. GSSG

Cultures containing matched levels of GSH or GSSG at 0–30 mM were initiated to determine whether the redox state of glutathione was im-

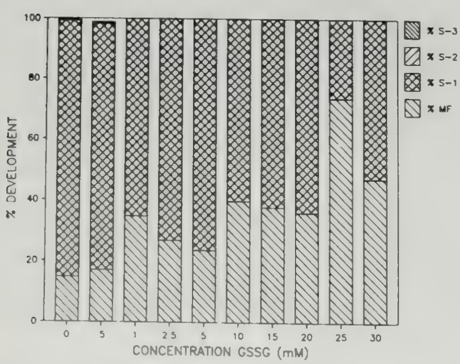

FIGURE 5. Developmental profile of microfilariae cultured in L-15 medium containing 10% FBS and supplemented with 0–30 mM GSSG. Values were derived from 3 experiments.

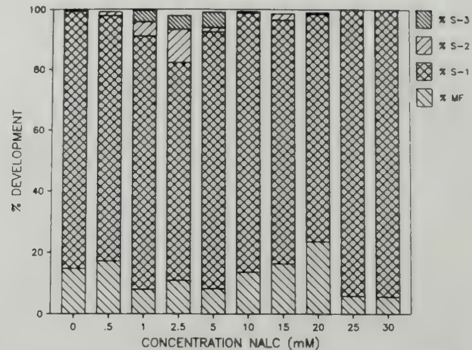

FIGURE 6. Developmental profile of microfilariae cultured in L-15 medium containing 10% FBS and further supplemented with 0–30 mM N-acetyl-L-cysteine. Values were derived from 8 experiments. Mean angular values representing development to the S-3 form at all tested concentrations of NALC were used in calculating 1-way analysis of variance ($F = 9.2494$, df1 = 12, df2 = 48, $P < 0.001$). Retransformed percentage data over the range of NALC that promoted development to the S-3 form (0.5–10 mM) were employed for computer generation of the second-order polynomial equation of $Y = 2.010 + 0.626X - 0.081X^2$ (R-value = 0.72).

portant in eliciting the response. GSSG did not enhance development at the concentrations tested (Fig. 5).

Cysteine delivery agents/analogues

To determine whether GSH functions as a cysteine delivery agent in this system, the intracellular cysteine delivery agents and analogues NALC, THIO, OTC, SMLC, DGLC, and CEE were each tested at standard concentrations against GSH controls. Results of tests with THIO and CEE were essentially similar as for CYS and DTT, whereas OTC and SMLC had little or no effect on development, following the same pattern as GSSG (data not shown). NALC and DGLC promoted notable development to the S-2 and S-3 forms at concentrations of 0.5–5 mM and 2.5–10 mM, respectively (Figs. 6, 7).

GSH vs. component amino acids

To ascertain whether the growth-promoting effects of GSH can be attributed to 1 or more of the component amino acids, the tripeptide was tested against groups containing 2.5 mM each of GLU, CYS, and GLY, or of GLN, CYS, and GLY. Both GLU and GLN were tested here due to the structural similarity of these amino acids. Component amino acids at the tested concen-

trations had no effect relative to unsupplemented controls (data not shown).

GSH depletion

To ascertain whether BSO, a sulfoximine inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979), or γGG, an inhibitor of γ-glutamyl transpeptidase (Anderson and Meister, 1986), could reverse or block the effect of GSH or NALC, BSO and γGG were each tested at 0–35 mM and at these concentrations in conjunction with 15 mM GSH or 1 mM NALC (BSO only). Neither BSO nor γGG alone or in conjunction with GSH or NALC had any effect relative to the respective unsupplemented GSH and NALC controls (data not shown).

Time exposure

To determine whether GSH functions as an early signal to trigger development, microfilariae were incubated in medium containing 15 mM GSH for the initial 0, 2, 24, and 168 hr of the 168-hr culture period. Exposure of microfilariae to GSH-supplemented medium for the initial 2 or 24 hr of culture did not result in enhancement compared to those in unsupplemented medium (data not shown).

DISCUSSION

The results presented here confirm the earlier finding (Devaney and Howells, 1983) that GSH enhances both the degree and frequency of development of *O. lienalis* microfilariae in this culture system. We further demonstrate that a dose-related response exists, that lower concentrations of FBS may be used than in previously cited studies, that neither TPB nor a feeder layer of cells is necessary for microfilarial development, and that specific cysteine analogues also elicit a dose-related developmental response.

Microfilariae of many *Onchocerca* spp., including *O. lienalis*, invade the thoracic flight musculature of adult *Simulium* spp., becoming intracellular parasites at this site before development proceeds (Hawking and Worms, 1961). It is noteworthy that the optimum levels of GSH for development of *O. lienalis* microfilariae *in vitro*, as determined here, conform rather closely to intracellular concentrations of GSH (0.5–8 mM) in nearly all organisms, including mosquitoes (Richie et al., 1987).

Intracellular glutathione is normally over 99% GSH (Meister and Anderson, 1983). Only this

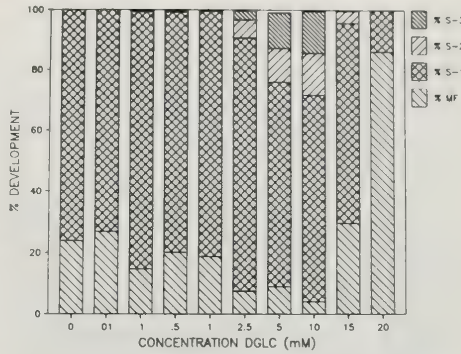

FIGURE 7. Developmental profile of microfilariae cultured in L-15 medium containing 10% FBS and supplemented with 0–20 mM D-glucose-L-cysteine. Values were derived from 4 experiments. Mean angular values representing development to the S-3 form at all tested concentrations of DGLC were used in calculating 1-way analysis of variance ($F = 14.1468$, df1 = 9, df2 = 19, $P < 0.001$). Retransformed percentage data over the range of DGLC that promoted development to the S-3 form (2.5–15 mM) were employed for computer generation of the second-order polynomial equation of $Y = -4.219 + 3.412X - 0.207X^2$ (R-value = 1).

reduced form led to any significant development in our culture system. Although GSH has reductive properties, other reducing agents (CYS and DTT) did not stimulate development. This led us to investigate other properties of the GSH molecule.

CYS is required for the synthesis of proteins, glutathione, and other compounds. CYS has been shown to be toxic in tissue culture systems (Nishiuch et al., 1976) and is rapidly oxidized to form the insoluble cystine (Meister et al., 1986). In contrast to the observations of Devaney and Howells (1983), CYS did not promote microfilarial development in our cultures.

Because glutathione serves as a storage form of CYS, the availability of CYS can be increased by use of procedures that increase glutathione levels. Glutathione is not effectively transported into cells; it is broken down extracellularly by membrane-bound γ-glutamyl transpeptidase and dipeptidase activities. Resulting products may then be transported into cells and act as substrates for intracellular glutathione synthesis (Meister et al., 1986). CYS precursors and analogues were therefore administered in our cultures in attempts to increase the availability of cysteine for glutathione synthesis. NALC is thought to be deacetylated *in vivo* to cysteine,

and acts indirectly to replenish glutathione by providing this limiting precursor (Meister et al., 1986; Wong and Corcoran, 1987). NALC and DGLC did enhance microfilarial development, thus supporting the view that NALC and possibly DGLC were acting as GSH precursors in this system.

In contrast to NALC and DGLC, no other precursors or analogues tested enhanced microfilarial development. SMLC, a cysteine analogue that lacks a free sulfhydryl group, did not support microfilarial development. Although CYS and CEE both contain free sulfhydryl groups, neither enhanced microfilarial development. Oxidation and/or de-esterification reactions promptly render these molecules inactive in a culture environment. THIO is known to be converted to N-formylcysteine (Debey et al., 1958), however, this compound inhibited microfilarial development in culture. OTC has been shown in other systems to be an excellent substrate for 5-oxoprolinase, which converts it to CYS, subsequently leading to increased intracellular glutathione levels (Williamson and Meister, 1981). OTC administration had no apparent effect on microfilarial development.

In further attempts to ascertain whether intracellular uptake or (re)synthesis of GSH influenced the observed differentiation of microfilariae in culture, inhibitors of γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase were administered to microfilariae in culture. Neither BSO nor γGG administration depressed the GSH-dependent development by microfilariae. Assuming that these enzyme inhibitors could access their respective sites, this suggests that GSH either modifies the culture environment or acts as a signal to stimulate development. Enhanced development due to culture environment modification is most probable in light of the failure of the 24-hr pretreatment of microfilariae with GSH to promote development.

Addition of the component amino acids of GSH did not lead to enhanced development. GSH is generally believed to partially dissociate *in vitro* to dipeptide forms; that such products might be the active moieties of GSH influencing microfilarial development warrants further investigation. Specific di- and tripeptide fragments unrelated to GSH have been shown to have a stimulatory effect on the growth of *Caenorhabditis briggsae* (Pinnock et al., 1975).

The 3 compounds tested that do enhance development by *O. lienalis* microfilariae (GSH, NALC, and DGLC) are all cysteine analogues with a free sulfhydryl group. GSH, NALC, and presumably DGLC will inactivate reactive compounds either by reduction or conjugation. GSH-dependent reduction of hydroperoxides and GSH conjugation of electrophiles are generally catalyzed by glutathione reductase and glutathione transferase, respectively, whereas NALC-dependent reactions are nonenzymatic in nature. Interactions of GSH or NALC with free-radical intermediates are not enzyme catalyzed and are thought to be of considerable physiologic importance (Moldeus et al., 1986). These observations suggest that reduction of hydroperoxides and conjugation of electrophiles may be important to microfilarial development in a culture environment.

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POPULATION DYNAMICS OF *DISTOICHOMETRA BUFONIS* (CESTODA: NEMATOTAENIIDAE) IN *BUFO WOODHOUSII*

E. L. Hardin and J. Janovy, Jr.
School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588-0118

ABSTRACT: Prevalence, density, and variance/mean (=variance/density) ratios are reported for the cestode *Distoichometra bufonis* in 28 samples of the amphibian host, *Bufo woodhousii*, taken over a 3-yr period at 2 study sites on the South Platte River in Keith County, Nebraska, U.S.A. In addition, changes in host demography resulting from entry of newly metamorphosed toads into the terrestrial population are given. Prevalence and density (average number of worms per host, infected + noninfected) did not vary significantly either within or between sites and years unless newly metamorphosed toads were included in the analysis. Prevalence ranged from 70 to 100%, and density from 2.7 to 14.8 worms per host, excluding samples containing newly metamorphosed toads. The bulk of metamorphosis occurred in late June or early July, and by August both prevalence and density had returned to their premetamorphosis values. The host/parasite system is interpreted as one in which disruption of the host population's demographic makeup only temporarily perturbs parasite population structure.

The Rocky Mountain toad, *Bufo woodhousii* Girard, 1854, occurs throughout most of Nebraska. Casual observations from 1976 to 1983 suggested that the toad had a relatively short breeding season in western Nebraska (late May and June), that breeding took place in ephemeral ponds on a schedule that produced a rather synchronous influx of newly metamorphosed individuals into the terrestrial population, usually in early July, and that prevalence of the nematotaeniid cestode, *Distoichometra bufonis* Dickey, 1921, was quite high. The studies reported here were done in an attempt to describe some of the population level interactions between the toads and these cestodes.

As a resource for *D. bufonis, B. woodhousii* presents 2 interesting problems: (1) physically, the host is a patchy and ephemeral resource in the sense of Price (1980), especially because the toads are in a state of torpor during the winter, and (2) the host population is disrupted demographically with the influx of newly metamorphosed toads, a perturbation that alters the resources available to the parasites. Thus, even though the life cycle of *D. bufonis* is not known, parasite population dynamics should reveal, in part, the manner in which the worm population structure is maintained in nature. The study was conducted by attempting to answer the following specific questions: (1) Does the demographic makeup of the host population change over the course of the active season? (2) In what manner are the parasites distributed among the hosts? (3) Are the observed parasite distributions consistent between years and sites? (4) At what stage in the host's life does it become infected? (5) Are the hosts exposed to infections throughout the active season? (6) Does there exist a relationship between parasite density and prevalence, and host size (approximate measure of age) and sex? And (7) does the parasite overwinter in the definitive host?

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MATERIALS AND METHODS

The host species was identified as *Bufo woodhousii* using Ballinger and Lynch (1983). Tadpole specimens were identified using larval *Bufo* characters of Wright and Wright (1949) and identification of toads produced from these tadpoles. The parasite species was identified as *D. bufonis* using original descriptions by Dickey (1921) and of *D. kozloffi* by Douglas (1958), as well as *D. bufonis* types borrowed from the USNM Helminthological Collection, Beltsville, Maryland, and Nebraska specimens borrowed from the H. W. Manter Laboratory, University of Nebraska State Museum.

Study sites

Two sites were used, both near the South Platte River in Keith County, Nebraska. The river generally flows at capacity in the spring due to snowmelt runoff. During May and early June, receding waters and spring rains contribute to the formation of ephemeral ponds, the main breeding habitat of *B. woodhousii*. The primary study site used from 1984 to 1986 was a rectangle 300 × 500 m, near an abandoned gravel pit 4 km east of the town of Roscoe, Nebraska. A second site, chosen for its similarity to the Roscoe one, was 1 km south of Brule, Nebraska, and was used in 1986. One site, and the year it was used, is defined as a site-year; the study thus covered 4 site-years (Table I).

Sampling

The breeding of *B. woodhousii* near the South Platte River is relatively synchronous. Toads mate in late May and early June and most tadpoles metamorphose

during a 2- or 3-wk period in late June or early July. Collecting began in mid-April except in 1984 when it began in June, and ended in late September or early October. Adult toads were collected biweekly at each study site over the course of the active season. Tadpoles were sampled once a week when available.

Hosts were taken to the Cedar Point Biological Station 13 km north of Ogallala, Nebraska, and dissected within 24 hr of capture. Snout/vent length (SVL) and sex of each toad were recorded. Number of worms per individual host was determined by scolex count. All tissues of tadpoles were examined for parasites.

Recruitment rates were measured by use of sentinal toads and mark–recapture studies. In the sentinal study, during 1985, 200 newly metamorphosed toads (16–21 mm SVL) were collected from the north shore of Lake McConaughy in an area in which no adults were found to be infected with *D. bufonis*. Sixty toads were dissected to ensure the population was initially uninfected. The remaining 140 toads were split into 2 groups of 70 and each group placed in an 18-m^2 enclosure that had been cleared of indigenous toads, on the banks of an ephemeral pond, in the study site. Both groups were exposed to the area of natural transmission for 2 hr in the early evening. One group was returned for additional 2-hr periods on 6 consecutive days, for a total of 14 hr of exposure. The stomach contents of 5 toads from each group were collected by dissection on site and saved in 70% ethanol. In the mark–recapture study in 1986, 170 adult toads of various sizes were collected from the north shore of Lake McConaughy. Fifty were dissected to confirm that the group did not harbor *D. bufonis*. The remaining 120 were toe-clipped and released at Roscoe on 28 July. Marked toads were recaptured on 4 August and 18 August. In both studies, recruitment rates were expressed as number of worms acquired per toad per unit time exposure. A cohort from the original host population was dissected after the studies to ensure the toads were still free of *D. bufonis*.

Attempts to find *B. woodhousii* in the winter were unsuccessful. However, 6 toads from the Roscoe site were determined to be infected by the presence of *D. bufonis* proglottids, parauterine organs, and egg capsules in the feces. These toads were then placed in moist sand in an aquarium, which was put into a refrigerator whose temperature was cooled to 4 C over a 24-hr period. Six weeks later the aquarium was removed and the toads examined for worms.

Statistical analysis

Young-of-the-year, second-year, and older-than-second-year groups were established from SVL distributions, with 65 mm being chosen as the point separating the latter 2 groups. This choice was based on measurements from 1985 and 1986. In the first sample from each site, no toads between 57 and 66 mm were found, whereas numerous toads between 39 and 57 mm, and greater than 66 mm SVL were collected.

Density and variance/density ratios were analyzed by the methods of Taylor et al. (1978). Contingency table analysis was used to determine if prevalence was independent of host sex and size. Relationships between infrapopulations and host size were determined from regression lines using SVL and number of worms for the total collection and demographic subgroups. Differences between samples within site-years, and between site-years, were analyzed by means of the Kruskal-Wallis nonparametric method (Sokal and Rohlf, 1981) using infrapopulations as the observations to be ranked.

Terminology is consistent with that recommended by Margolis et al. (1982). Density is defined as the average number of worms per toad (infected + noninfected) in a sample.

Table I. *Sampling data and parasite distribution parameters for* D. bufonis *in* B. woodhousii.

Site-year	Sample date	n	Mean SVL	Prevalence (%)	Density	Variance/density
Roscoe, 1984	6/20*	7	35.4	57	4.0	5.8
	7/06	14	41.1	79	6.1	6.4
	7/16	12	44.0	75	9.6	7.7
	7/30	14	39.7	100	11.9	5.5
	8/14	10	50.8	100	10.8	6.6
	8/22	8	44.8	100	11.9	4.4
	9/12	11	46.7	91	9.7	3.3
Roscoe, 1985	5/08	20	47.1	70	6.2	5.9
	5/28	16	46.8	81	7.4	7.4
	6/11	14	49.9	71	2.7	2.7
	6/24	10	53.6	100	6.1	3.7
	7/07*	10	37.9	80	6.2	5.4
	7/20	15	33.3	53	3.9	4.9
	8/02	11	35.2	91	6.2	2.7
	8/16	9	37.9	89	14.8	8.8
	9/15	15	45.6	73	8.3	5.9
Roscoe, 1986	5/12	16	65.6	100	10.8	8.2
	6/02	20	54.7	90	5.7	3.0
	6/19	16	67.1	82	6.1	7.2
	7/05*	24	36.8	46	2.3	5.0
	7/26	40	29.8	40	1.8	5.5
	8/18	20	38.3	85	4.5	3.3
Brule, 1986	5/14	22	50.7	86	4.4	3.4
	6/01	20	48.9	75	4.6	3.8
	6/23	18	57.9	78	4.2	3.0
	7/05*	25	30.5	28	1.6	7.2
	7/27	40	37.0	45	1.9	3.6
	8/16	20	38.5	85	6.3	6.5

* Sample dates on which newly metamorphosed toads first appeared in the terrestrial population.

RESULTS

Collection dates, sample sizes, mean host SVL, worm prevalences, densities, and variance/density ratios are given in Table I. No toads were collected on trips to Roscoe in 1985 and 1986, and to Brule in 1986, prior to the first samples listed, or on trips to the study sites 3 wk after the last sample of each site-year. The annual drop in SVL seen in Table I was due to metamorphosis of tadpoles. Roscoe, in 1984, did not include premetamorphosis toads, but the slow increase in SVL was similar to that of postmetamorphosis samples in other years.

TABLE II. *Relationships between snout/vent length and* D. bufonis *infrapopulations.*

Site-year	Group	Slope	Y-intercept	r	Variance due to regression	Variance about regression	F	df
Roscoe,	first yr	0.41	−1.84	0.34*	331.41	64.76	5.12†	1,41
1984	second yr+	−0.15	15.89	−0.32*	99.95	26.90	3.72	1,31
Roscoe,	first yr	0.37	−4.82	0.31*	268.88	54.30	4.95†	1,47
1985	second yr+	−0.08	9.79	−0.15	53.31	33.91	1.57	1,69
Roscoe,	first yr	0.15	−2.97	0.35*	75.75	7.71	9.82†	1,72
1986	second yr+	0.03	5.44	0.08	17.74	46.42	0.38	1,61
Brule,	first yr	0.21	−3.61	0.39*	213.56	16.84	12.68†	1,72
1986	second yr+	−0.05	6.83	−0.12	16.14	15.05	1.07	1,69

* The probability of obtaining higher absolute r-values by chance alone if SVL is independent of infrapopulation is less than 0.05.

† The probability of obtaining observed F-value by chance alone if variances are equal is less than 0.05.

Parasite populations were overdispersed in all samples (Table I). Densities and prevalences were generally at their lowest during and shortly after the entry of newly metamorphosed toads into the terrestrial population, then approached those of the premetamorphosis period within a few weeks. Kruskal-Wallis analysis showed no difference in "density location" (see Sokal and Rohlf, 1981) for Roscoe, 1984, and Roscoe, 1985, samples, or for the 4 site-years when samples within site-years were combined. The 2 site-years for 1986 yielded an adjusted Kruskal-Wallis H-value high enough to reject the hypothesis of no difference between sample infrapopulations, but removal of the samples with newly metamorphosed toads reduced the adjusted H-value enough to accept the null hypothesis. Thus, the distribution of *D. bufonis* infrapopulations in toad samples was essentially the same for all collections unless, in some cases, a known alteration in the demographic makeup of the host population occurred. In such cases, removal of the heterogeneous samples allowed acceptance of the null hypothesis for all the remaining samples.

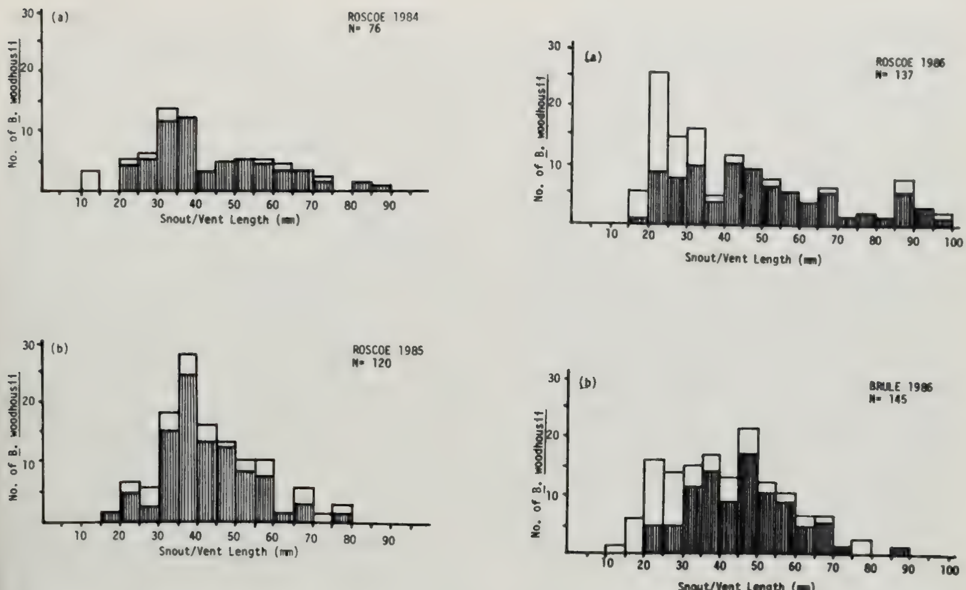
With the exception of newly metamorphosed toads, virtually all size classes of hosts were infected (Figs. 1, 2). However, the prevalence among 15–25-mm SVL toads depended somewhat on the year (Roscoe, 1984, 4/8 infected; Roscoe, 1985, 2/8; Roscoe, 1986, 10/34; Brule, 1986, 5/24). Thus, recruitment began within a few days after metamorphosis and continued during the first 2 mo of terrestrial life. Significant positive correlations were obtained between young-of-the-year SVL and infrapopulation, but similar results were not observed when older toads were included in the analysis or analyzed separately (Table II). Recruitment was probably not confined to the young of the year, however, because 6 larger toads were found with short translucent worms typical of those in small young-of-the-year hosts. Increases in density were accompanied by increasing overdispersion (Table III). Contingency table analysis showed prevalence to be independent of sex when all samples were combined. Prevalence was not independent of size, with the second-year toads being most frequently infected (Table IV).

In the recruitment experiments, no infections were found in small toads allowed to feed for 2 and 14 hr. The feeding rates were 5.8 and 5.1 prey items/hr/toad for the 2 groups, respectively. In the mark–recapture study, 1 of 6 toads recovered 1 wk after release was infected with 5 immature worms, and 3 additional toads recovered 3 wk after release were all infected. The

TABLE III. *Log variance/log density relationships in the* D. bufonis/B. woodhousii *system.*

Site-year	Slope	Y-intercept	r	Variance due to regression	Variance about regression	F	df
Roscoe, 1984	0.85	2.02	0.78	0.73	0.10	7.30	1,5
Roscoe, 1985	1.62	0.49	0.89	3.53	0.13	27.81	1,7
Roscoe, 1986	1.16	1.38	0.89	2.93	0.16	15.31	1,4
Brule, 1986	0.86	1.64	0.79	1.10	0.16	6.84*	1,4

* The probability of obtaining the observed F-value by chance alone if variances are equal is less than 0.10. For all other F-values the probability is less than 0.05. All r-values are high enough to reject the hypothesis that log variance is independent of log density, at the 0.05 level.



FIGURES 1, 2. Distribution of *B. woodhousii* by size for 4 site-years, and distribution of the infected subpopulations (shaded portions of the bars).

cohorts of these experimental animals, on the north shore of Lake McConaughy, remained free of worms.

Out of 400 tadpoles and 125 newly metamorphosed toads dissected, none were found to have worms either in the intestinal lumen or viscera.

All 6 toads refrigerated for 6 wk were found to be infected with living *D. bufonis,* but some of the worms had evidently aborted their strobilae. The short worms were relatively opaque, unlike the translucent ones, of similar length, in young of the year.

DISCUSSION

The results suggest that a parasite's population *structure,* as described by distribution parameters, can be regulated in certain host/parasite systems in nature. Thus, in this natural experiment (see Diamond, 1986), the introduction of large numbers of uninfected toads into the host population produced only temporary changes in prevalence, density, or variance/density ratios in *D. bufonis.* The general stability of the parasite population is shown by the fact that over the 4 site-years, prevalence varied between 70 and 100%, density between 3 and 15, and variance/density ratios between 3 and 9, when samples containing newly metamorphosed toads were excluded. Furthermore, the results of the Kruskal-Wallis tests revealed no significant differences in "location" of infrapopulations between samples within years, and between years, again excluding the samples with newly metamorphosed toads.

Although the variations reported for density and prevalence may seem substantial, they include sampling error but are still within a single order of magnitude, unlike the population structure variation seen in some other systems sam-

TABLE IV. *Contingency table analysis of prevalences by sex and age (estimated from SVL), of* D. bufonis *infections in* B. woodhousii.

Group	Number infected	Number noninfected	Chi-square
Males	99	17	
Females	105	26	1.15
Young of the year	141	98	
Second year	155	23	39.06*
Second year	155	23	
Third year	46	14	3.71
Young of the year	141	98	
Third year	46	14	6.39*

* Chances of obtaining chi-value by chance alone less than 0.05 if frequency of infection is independent of compared demographic group.

pled repeatedly. For example, Jarroll (1979) reported variance/mean ratios from 4.7 to 51.7 with *Bothriocephalus rarus* in newts, and Grimes and Miller (1976) reported ratios of 0.66–92.38 with *Monobothrium ulmeri* in *Erimyzon oblongus*. A number of other parasite species' populations exhibit density and variance/density ratios that fluctuate over 2 or more magnitudes (Lemly and Esch, 1984; Janovy and Hardin, 1987). In addition, prevalence is high during the toads' active season, which, along with the low and fairly stable density, suggests that most of the *D. bufonis* genetic variation is displayed against most of the heterogeneity provided by the definitive host. That is, parasites are not aggregated into a few host individuals. This basic feature of the relationship is achieved through rather indiscriminate transmission to young of the year coupled with an as yet unknown mechanism for limiting the truly heavy infections that are the basis for high variances in many other systems.

The slopes of regression lines describing the relationship between worm population distribution log density and log variance are about half those reported for some other host/parasite combinations. (Taylor et al. [1978]; Jarroll [1979], 2.8* for *B. rarus* in newts; Lemly and Esch [1984], 2.2* for *U. ambloplitis* in sunfish; Janovy and Hardin [1987]; Riggs and Esch [1987], 2.3* and 3.8* for *B. acheilognathi* in 2 cyprinid species. * = Slopes not actually given in the published paper, but calculated from data in the tables or figures.) This observation means that mechanisms that produce overdispersion in the *D. bufonis/B. woodhousii* system do not operate strongly to link increasing density with exponentially increasing variance.

The results provide definitive answers to most of the original questions, and in so doing, describe the parasite population's behavior that characterizes natural transmission. The infection is primarily one of first- and second-year toads, although that distribution may be as much of ecological as physiological origin, because the intermediate host, if present, is small enough to be eaten by a 20-mm SVL toad, and eaten in fairly large numbers. For example, if the sentinel toad experiments can be extrapolated to the marked and recaptured toads, then an individual *B. woodhousii* consumes at least 4,000 prey items per cestode infection.

The life cycle of *D. bufonis* is unknown. Joyeux (1924) and Stumpf (1981/1982) both stated that the life cycle was direct in *Cylindrotaenia* sp., a related nematotaeniid. Prudhoe and Bray (1982) did not find the Joyeux (1924) work completely convincing, and our own efforts to repeat the Stumpf (1981/1982) experiments were not successful. No infections were produced in newly emerged toads, *Tenebrio molitor*, or *Periplaneta americana* force-fed proglottids, although egg capsules could be found in the intestine. Nor could ingested eggs be found in the guts of lab-raised *Tribolium confusum*, or wild-caught carabids (2 species) or staphylinids (frequent prey items for small *B. woodhousii*) exposed to macerated proglottids on moist filter paper. Although these experiments were not exhaustive, their failure suggests that the problem of nematotaeniid life cycles is far from being solved.

However, the life cycle is not only easily completed under suitable ecological conditions in nature, it is just as easily interrupted for long periods by unsuitable conditions. The latter is shown by the persistence of noninfected *B. woodhousii* populations on the north shore of Lake McConaughy (Keith County, Nebraska). The breeding biology of *B. woodhousii*, and by inference that of other species, as well as the ecological circumstances under which they exist at Lake McConaughy, are determined by surface-water storage and use policies of the Central Nebraska Public Power and Irrigation District. At the South Platte River study sites, these aspects of toad life are determined largely by Rocky Mountain snow pack. Thus, shoreline physical characteristics have evidently rendered *D. bufonis* locally extinct on Lake McConaughy, possibly through their effects on an intermediate host. Anecdotal evidence and student collections from 1975 to 1979 indicate that the worm did occur in toads from Lake McConaughy prior to the study reported in this paper. *Distoichometra bufonis* specimens 21042 and 21043, H. W. Manter Laboratory, UN State Museum, were collected from *B. woodhousii* at Lake McConaughy in 1979.

As a system, the *D. bufonis/B. woodhousii* pair is very similar to the *Gyrocotle* sp./*Chimaera monstrosa* system reviewed by Williams et al. (1987) in the sense that the worm populations are not only rather evenly distributed among virtually all demographic classes of the host population, but also evidently subject to infrapopulation regulating mechanisms that inhibit the accumulation of large numbers of parasites. Although infrapopulations of *D. bufonis* are not regulated to the same degree as those of gyro-

cotylideans, only 10 out of the 477 toads dissected in this study had 25 or more parasites. Thus, the Williams et al. (1987) description of the "general picture" of *Gyrocotyle* sp. populations as ". . . very high prevalence linked to low intensity . . ." applies also to the present study. It remains to be determined, of course, whether such population structures, as opposed to highly overdispersed ones with extreme variations in density and prevalence, are associated with genetically determined parasite traits.

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FIELD INVERSION GEL ELECTROPHORETIC SEPARATION OF *CRYPTOSPORIDIUM* SPP. CHROMOSOME-SIZED DNA

Jan R. Mead, Michael J. Arrowood, William L. Current*, and Charles R. Sterling†
Departments of Microbiology and Immunology, and Veterinary Science, University of Arizona, Tucson, Arizona 85721

ABSTRACT: Chromosomal DNA from 5 isolates of *Cryptosporidium parvum* and 1 of *C. baileyi* were compared by field-inversion gel electrophoresis (FIGE). FIGE analyses of parasite DNA prepared from purified sporozoites versus intact oocysts showed no observable differences. Chromosomal DNA migration patterns of the 5 *C. parvum* isolates were indistinguishable, whereas similar but distinct differences were evident between *C. baileyi* and the isolates of *C. parvum*. Oocyst-reactive monoclonal antibodies differentiated oocysts of *C. parvum* from those of *C. baileyi* but were unable to distinguish oocysts of 1 isolate of *C. parvum* from another.

Cryptosporidium spp. are important causes of diarrheal disease in man and several species of animals (Fayer and Ungar, 1986). In humans, the organism has been responsible for illness among travelers (Jokipii et al., 1985; Soave and Ma, 1985; Sterling et al., 1986), children in day-care centers (Centers for Disease Control, 1984; Taylor et al., 1985; Wiedermann et al., 1985), and immunocompromised individuals (Sloper et al., 1982; Current, 1985; Lewis et al., 1985). It has also been associated with 2 waterborne outbreaks of diarrheal illness (D'Antonio et al., 1985; Dennis Juranek, Centers for Disease Control, pers. comm.).

One major question regarding cryptosporidial infections of mammals is whether there is isolate variation. Morphological and life cycle differences were not observed among 3 isolates of *C. parvum* infecting Swiss Webster mice (Current and Reese, 1986). Virulence differences, however, have been observed in 2 isolates infecting calves (Fayer and Ungar, 1986).

One novel approach that has been used to demonstrate strain and species differences among clinical isolates of protozoa is pulsed-field gel electrophoresis (PFGE) of chromosomes (Van der Ploeg et al., 1984, 1985; Holmes Giannini et al., 1986). A modification of this technique, field-inversion gel electrophoresis (FIGE), has recently been used to separate yeast chromosomes (Carle et al., 1986). An adaptation of the FIGE technique was used in this study to investigate potential isolate and species chromosome size variations among 5 isolates of *C. parvum* and 1 of *C. baileyi*.

MATERIALS AND METHODS

Oocyst isolates

Five isolates of *Cryptosporidium parvum* were obtained for examination. Three isolates from calves were obtained from: (1) Dr. Harley Moon (NADC, Ames, Iowa), (2) Dr. Philip Klesius (USDA, Auburn, Alabama), and (3) the University of Arizona dairy farm (Tucson, Arizona). One isolate from horses was obtained from Dr. Thomas Klei (Louisiana State University, Baton Rouge, Louisiana). One human isolate was obtained from a traveler returning from Mexico (Sterling et al., 1986). Oocysts of a chicken isolate of *Cryptosporidium baileyi* were purified from infected chicken eggs according to the method of Lindsay et al. (1988).

Oocyst production

Oocysts of each isolate of *C. parvum* were used to infect 2-day-old Holstein calves (10^8/animal). Following the onset of oocyst shedding, feces were collected daily, mixed with an equal volume of 5% $K_2Cr_2O_7$, and stored at 4 C. Feces were sieved sequentially through stainless steel screens with a final mesh size of 230 (63 μm porosity).

Oocyst and sporozoite purification

Oocysts and sporozoites were purified using discontinuous sucrose and isopycnic Percoll (Pharmacia, Piscataway, New Jersey) gradient centrifugation techniques (Arrowood and Sterling, 1987). Briefly, sieved feces were centrifuged over 2 sequential discontinuous sucrose gradients prepared using 0.025 M phosphate-buffered saline, pH 7.2 (PBS), and 1% Tween 80. Oocyst-enriched bands were recovered, pooled, and layered over Percoll (1.091 g/ml) in high-speed centrifuge tubes and centrifuged at 22,000 *g*. Recovered oocysts were stored at 4 C in 2.5% $K_2Cr_2O_7$ or washed with PBS and suspended in excysting solution (0.25% [w/v] trypsin, 0.75% [w/v] sodium taurocholate in PBS) at 37 C for 60 min. The excystation mixture (oocysts, oocyst walls, and sporozoites) was washed with Alsever's so-

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* Lilly Research Laboratories, A Division of Eli Lilly and Company, P.O. Box 708, Greenfield, Indiana 46140.

† To whom correspondence should be addressed.

lution, layered over Percoll, and centrifuged as described for oocysts. Sporozoites recovered from the Percoll gradients were washed 3 times with PBS.

Preparation of oocysts or sporozoites for FIGE

The following method was adapted from the trypanosome treatment method of Van der Ploeg et al. (1984). Percoll-isolated sporozoites ($3–5 \times 10^9$) or oocysts ($1–2 \times 10^9$) were washed with suspension buffer (75 mM sodium phosphate, 65 mM NaCl, 1% glucose), pH 8.0, and resuspended in 1 ml suspension buffer. The sporozoite or oocyst suspension was mixed with an equal volume of 1% low gelling temperature agarose (SeaPlaque, FMC BioProducts, Rockland, Maine) previously melted in suspension buffer and cooled to 37 C. Agarose suspensions were dispensed into sample holders in 100-μl aliquots and cooled on ice for 2 min. Each preparation of solidified agarose blocks was transferred to 10 ml cell lysis solution (0.5 M EDTA, 1.0% sodium N-lauroylsarcosine, 2.0 mg/ml proteinase K), pH 9.5, and incubated at 50 C for 48 hr. Following lysis treatment, agarose blocks were stored at 4 C in the lysis solution until used.

Agarose blocks of *Saccharomyces cerevisiae* were prepared following the method of Schwartz and Cantor (1984). Purified lyticase (L8137, Sigma Chemical Co., St. Louis, Missouri) was substituted for zymolase. Approximately 10 units of lyticase were employed per ml of yeast cells (1.2×10^{10} cells). Treated agarose blocks were stored at 4 C in the lysis solution until used.

Agarose gel electrophoresis

DNA was electrophoresed using a horizontal Sub Cell DNA electrophoresis cell (Bio-Rad Laboratories, Richmond, California) after the method of Carle et al. (1986). Resolving gels (1% agarose) were prepared by melting agarose (GTG grade, FMC BioProducts) in 45 mM Tris, 45 mM boric acid, 1.25 mM ethylenediamine tetraacetic acid buffer, pH 8.0 (TBE buffer), cooling to 65 C, and pouring into a 15×25-cm casting tray (200 ml). Agarose blocks were cut in half and placed in 1-cm slots of the solidified agarose resolving gel. Precooled TBE buffer was maintained at 11 C by circulating through a cooling cell (submerged in a refrigerated water bath) before flowing through the electrophoresis cell. A constant voltage gradient of 10 V/cm was employed (250 V total) for each 24-hr run. The electric field was inverted throughout the run beginning with a forward field interval of 9 sec, which incrementally increased to 72 sec by the end of 24 hr. The initial 3-sec reverse interval alternated with the forward interval, incrementing to a final 24 sec. The resulting switching interval ramp was linear and maintained a 3:1 (forward : reverse) ratio.

Field switching and switching interval ramp generation were accomplished using a microcomputer (Commodore 64, Commodore Business Machines Inc., West Chester, Pennsylvania) interfaced with high-voltage relays (K42C332, Kilovac Corp., Santa Barbara, California). Software was written to allow convenient generation of precise switching interval ramps (the program is also suitable for nonramped pulsed-field applications). A circuit diagram and copy of the software are available on request.

Oocyst reactions with murine monoclonal antibodies

Two oocyst-specific monoclonal antibodies were used in immunofluorescent assays to label oocysts of the various isolates. The monoclonal antibody C1B3, an IgG1, was derived in our laboratory by immunizing BALB/c mice with oocyst walls purified from the Iowa isolate of *C. parvum* (Sterling and Arrowood, 1986). The monoclonal antibody OW3, an IgM, was derived in our laboratory by immunizing RBF/Dn mice with Percoll-purified oocyst walls from the Iowa isolate of *C. parvum*. Specificity of the OW3 monoclonal antibody for oocysts (and lack of cross-reactivity with other organisms) was verified by Garcia et al. (1987).

Briefly, monoclonal antibodies were applied to air-dried, heat-fixed fecal smears, incubated in a humid chamber for 20 min at room temperature, and then rinsed with PBS. Coverslips were then applied to slides treated with the C1B3 monoclonal antibody (previously conjugated with fluorescein isothiocyanate). A secondary antibody (goat anti-mouse IgG, IgA, IgM), conjugated with fluorescein isothiocyanate (American Qualex International Inc., La Mirada, California), was diluted 1:100 in PBS, applied to the smears, incubated for 20 min in a humid chamber, and rinsed with PBS. Slides were coverslip mounted and examined by epifluorescent microscopy.

RESULTS

Electrophoresed DNA of *C. parvum* is shown in Figure 1. Lanes 1–5 contain *C. parvum* isolates from Iowa (calf), Louisiana (horse), Arizona (calf), Mexico (human), and Alabama (calf), respectively. Five bands are demonstrated in each of the 5 isolates. The DNA bands range in size from approximately 1,400 kb for the smallest to over 3,300 kb for the largest. No distinct differences were observed in the migration pattern of the bands between mammalian isolates.

A diffusely stained band appeared at approximately 290 kb for some of the isolates (e.g., Fig. 1, lanes 3–5). Repeated experiments indicate that this region does not stain when purified sporozoites are prepared in agarose (Fig. 1, lane 2). Additionally, the region is not discernible if oocysts are purified immediately from infected calf feces and processed for FIGE analysis (Fig. 1, lane 1). Agarose-embedded oocysts of *C. baileyi* (freshly isolated from infected chicken eggs) also showed no staining in this region. Oocysts previously stored in 2.5% $K_2Cr_2O_7$ at 4 C for extended periods (6 mo) before processing for electrophoresis showed the strongest staining in the 290-kb region (Fig. 1, lanes 3–5).

Figure 1, lane 6, shows 6 bands of *C. baileyi*. The smallest band is approximately 1,400 kb and the largest over 3,300 kb. The migration pattern of the bands differed distinctly from the pattern

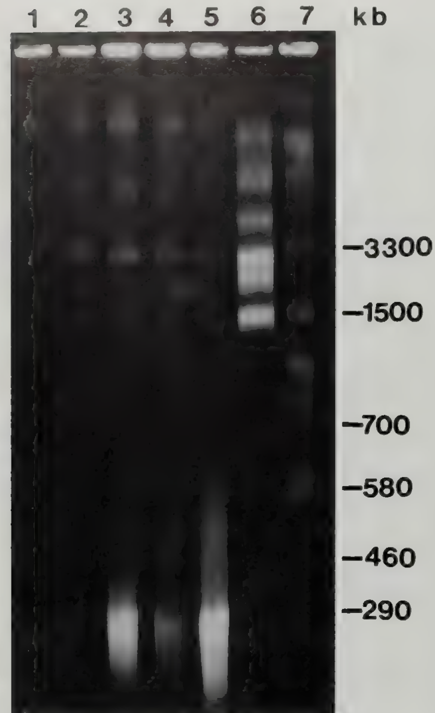

FIGURE 1. FIGE separation of chromosomal DNA's from *Cryptosporidium parvum* (lanes 1–5) and *C. baileyi* (lane 6) using a switching interval ramp. The forward-migration interval was linearly incremented from 9 sec at t = 0 hr to 72 sec at t = 24 hr with a constant 3:1 ratio between the forward and reverse intervals. Lane 1: purified oocysts (2 mo old) of the Iowa calf isolate; lane 2: purified sporozoites of the Louisiana horse isolate; lane 3: purified oocysts (6 mo old) of the Arizona calf isolate; lane 4: purified oocysts (6 mo old) of the Mexico human isolate; lane 5: purified oocysts (6 mo old) of the Alabama calf isolate; lane 6: purified oocysts (1 mo old) of the chicken isolate; lane 7: yeast.

displayed by all 5 *C. parvum* isolates. Two bands coincided with bands of *C. parvum,* whereas the 4 remaining bands did not specifically match any of those seen in the *C. parvum* isolates. Chromosomal DNA bands from *Saccharomyces cerevisiae* are shown in lane 7. The smallest band is approximately 290 kb and the largest is over 3,300 kb.

Table I illustrates the anti-oocyst monoclonal antibody labeling patterns for the isolates of *C. parvum* and *C. baileyi.* The monoclonal antibody C1B3 reacted with all isolates of *C. parvum* and with those of *C. baileyi.* The monoclonal antibody OW3 of *C. parvum* but not with

TABLE I. *Immunofluorescent reactivity of oocyst-specific monoclonal antibodies to oocysts of 5 isolates of* Cryptosporidium parvum *and 1 of* Cryptosporidium baileyi.

Isolate	Monoclonal antibody C1B3	Monoclonal antibody OW3
C. parvum		
Iowa	+	+
Louisiana	+	+
Arizona	+	+
Mexico	+	+
Alabama	+	+
C. baileyi	+	–

DISCUSSION

Pulsed-field gel electrophoresis of DNA, a relatively new technique, has been used to differentiate species and subspecies of *Leishmania* (Holmes Gianini et al., 1986; Scholler et al., 1986) and strains of *Plasmodium falciparum* (Kemp et al., 1985; Van der Ploeg et al., 1985). We have adapted the related technique, field-inversion gel electrophoresis (Carle et al., 1986), for use with the coccidian *Cryptosporidium.*

Two methods of preparing parasite DNA were compared to devise a rapid, but effective technique. Purified, naked sporozoites were embedded in agarose prior to digestion in lysis solution. For comparison, intact, purified oocysts were embedded in agarose before treatment with the lysis solution. Chromosomal banding patterns for the 2 preparations were essentially identical. We now routinely embed intact oocysts in agarose as the first step in preparing the DNA for FIGE and PFGE. It may be possible to directly embed oocysts of other species of coccidia (eliminating sporozoite purification) for FIGE and PFGE analysis. Directly embedding cysts of other protozoa may also be possible.

Some of the embedded oocyst preparations showed diffuse DNA staining at approximately 290 kb. We suggest that a sizeable portion of the embedded oocysts from these preparations are nonviable and contain degraded DNA that migrates to the 290-kb region. Speer and Reduker (1986) have shown an age-related decrease in oocyst excystation efficiency. In our laboratory, oocyst excystation rates decrease significantly upon extended storage. Freshly isolated oocysts generally excyst at rates of 90% or greater, whereas oocysts stored 6 mo or more rarely excyst at rates greater than 40–50%. The nonexcysting oocysts are presumably nonviable.

The FIGE technique has been shown to be valid for differentiating isolates of *C. parvum* from *C. baileyi*. These chromosomal DNA differences are probably translated into differences in cell surface proteins. Supportive evidence for this includes differential reactivity to oocyst antigens by monoclonal antibodies. The lack of oocyst antigen and chromosomal band differences among isolates of *C. parvum* suggest this species has a conserved genome. Size-independent differences in the DNA (sequence differences) may remain undetected by the FIGE technique. To investigate this possibility, 2-dimensional gel electrophoresis of sporozoite proteins is currently underway. This technique should detect differences in protein and antigen structure resulting from isolate variations in DNA sequences.

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KINETICS OF DEVELOPMENT OF INFLAMMATORY LESIONS IN MYOCARDIAL AND SKELETAL MUSCLE IN EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

Hugo A. Molina* and Felipe Kierszenbaum†
Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101

ABSTRACT: We studied the kinetics of development of inflammation in the myocardium and skeletal muscles of mice infected with *Trypanosoma cruzi* by determining the numbers of mononuclear cells (MNC), neutrophils, and eosinophils at tissue sites with varying degrees of damage. In the myocardium, areas with incipient inflammation and preserved tissue had the smallest numbers of inflammatory cells, 96–100% of which were MNC. In lesions where inflammatory cells accumulated in interstitial spaces displacing myofibers, MNC were also predominant (>98%) but were present in larger numbers than in areas with preserved tissue. The number of MNC was even larger in necrotic areas where there was also marked neutrophil infiltration at the time when amastigote nests were frequently present. In skeletal muscle, MNC were also the first cells to infiltrate lesion sites; their numbers increased with the degree of severity of the lesion. Neutrophil accumulation also accompanied skeletal muscle necrosis. A salient difference was eosinophil accumulation in the necrotic lesions of skeletal muscle but not in the myocardium. The results identify MNC as the cell that initiates the inflammatory process in the heart and skeletal muscles of *T. cruzi*-infected mice. In these tissues the number of MNC appeared to be a good correlate of lesion severity.

A number of reports have qualitatively described the cell types that infiltrate the inflammatory lesions in human (reviewed by Romaña, 1963; Andrade and Andrade, 1979; Andrade, 1985; Molina, 1986) and experimental Chagas' disease (Federici et al., 1964; Andrade and Grimaud, 1986; Bijovsky, reviewed by Andrade and Andrade, 1979 and by Molina, 1986). Yet, we know very little about the kinetics of development of these inflammatory lesions. In a recent study, we determined the numbers of different types of inflammatory cells in myocardial lesions of chagasic patients (Molina and Kierszenbaum, 1987). We were able to define the composition of cell infiltrates in lesions of varying degrees of severity but could not draw conclusions about the kinetics of development of inflammation because, working with human materials, there are many uncertainties, including the time of initiation of the infection. To circumvent these difficulties we used the mouse model system of Chagas' disease, which permits examination of both the acute and chronic stages (Hayes and Kierszenbaum, 1981). At various times postinfection (PI), we determined the numbers of inflammatory cells present in myocardial and skeletal muscle lesions of increasing degrees of severity.

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* Present address: Departamento de Neuropatología, Instituto de Investigaciones Neurológicas "Dr. Carrea," F.L.E.N.I., Ayacucho 2166, 1112 Buenos Aires, Argentina.

† To whom correspondence should be addressed at the Department of Biochemistry, New York University Medical Center, 550 First Avenue, New York, New York 10016.

MATERIALS AND METHODS

Animals

Four-week-old Crl-CD-1(ICR)BR Swiss mice (Charles River, Portage, Michigan) were used for parasite production. Four-week-old, inbred CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) were used for the histologic studies.

Infection

Trypanosoma cruzi, Tulahuén isolate, was used. Mice were infected i.p. with 1×10^5 trypomastigotes and bled 2 wk later from the retro-orbital venous plexus after ether anesthesia. The blood, collected into heparinized tubes, was diluted as necessary in RPMI-1640 medium containing 100 IU penicillin and 100 μg streptomycin per ml. Parasite concentrations were determined by a standardized microscopic procedure (Kierszenbaum and Saavedra, 1972). The CBA/J mice were infected i.p. with 20 trypomastigotes; control mice received medium alone.

Experimental design

Groups of 6 CBA/J mice were sacrificed at various times PI by sectioning of the axillary artery after ether anesthesia. Groups of 4 age-matched control mice were sacrificed at the same times. The heart, the quadriceps femoris, and the gastrocnemius muscles were removed and fixed in 10% formaldehyde in phosphate-buffered saline for 24 hr. The hearts were cut sagittally to examine the walls of the 4 cardiac chambers. The tissues

TABLE I. *Distribution of lesion types* in the myocardium and skeletal muscles at various times after* T. cruzi *infection.*

Day PI	Myocardium PT	Myocardium II	Myocardium DN	Skeletal muscle PT	Skeletal muscle II	Skeletal muscle DN
15	6/6†	6/6	3/6	6/6	5/6	2/6
30	6/6	6/6	4/6	6/6	3/6	3/6
45	6/6	5/6	4/6	6/6	4/6	4/6
60	6/6	2/6	0/6	6/6	3/6	0/6
90	6/6	1/6	0/6	6/6	2/6	0/6

* PT, tissue areas with minimal or no inflammation and preserved tissue; II, tissue areas showing interstitial inflammation with displacement of myofibers without necrosis or degenerative alterations; DN, tissue sites showing inflammatory infiltration with degeneration and necrosis of muscle fibers.

† No. mice with the indicated lesion type/no. mice examined.

were embedded in paraffin and 6-μm-thick sections were stained with hematoxylin and eosin Y. Three types of tissue areas were distinguished: those with minimal or absent inflammation and essentially preserved tissue (PT type); those presenting frequent interstitial inflammatory cell infiltrates with displacement of muscle fibers but no necrosis or degeneration of myofibers (II type), and those showing inflammatory infiltration associated with degeneration and necrosis of muscle fibers (DN type). The numbers of mononuclear cells (MNC), neutrophils (NEU), eosinophils (EOS), and amastigote nests in each of these types of areas were recorded.

TABLE II. *Type and distribution of inflammatory cells and amastigote nests in PT lesions of the heart.**

Day PI	Mouse no.	Severity of myocarditis	No. cells or amastigote nests per 100 fields: Mononuclear	Neutrophils	Eosinophils	Nests
15	1	+++	357	2	0	0
	2	+++	347	3	0	0
	3	++	233	3	0	0
	4	+	228	7	0	0
	5	+	200	1	0	0
	6	+	176	2	1	0
30	7	++++	250	0	1	0
	8	++++	257	10	0	0
	9	++++	237	9	0	0
	10	++	218	1	0	0
	11	+	177	0	1	0
	12	+	165	0	0	0
45	13	+++	309	4	1	0
	14	+++	371	0	0	0
	15	++	231	1	1	0
	16	++	196	1	1	0
	17	+	214	1	0	0
	18		179	1	0	0
60	19	+	285	2	1	0
	20	+	360	1	1	0
	21		456	3	0	0
	22	−	376	1	0	0
	23	−	357	1	0	0
	24		262	2	0	0
90	25	+	270	3	1	0
	26		222	2	0	0
	27		194	4	0	0
	28		193	3	0	0
	29	−	184	5	2	0
	30		158	1	0	0

* The average numbers of MNC, neutrophils, and eosinophils in myocardial tissue from uninfected age-matched mice did not vary significantly with age and were: 61 ± 16, 0.5 ± 0.2, and 0.3 ± 0.1, respectively.

TABLE III. *Type and distribution of inflammatory cells and amastigote nests in II lesions of the heart.*

Day PI	Mouse no.	Severity of myocarditis	No. cells or amastigote nests per 100 fields: Mononuclear	Neutrophils	Eosinophils	Nests
15	1	+++	926	6	2	0
	2	+++	846	6	1	0
	3	++	588	0	0	0
	4	+	798	3	1	0
	5	+	790	0	1	0
	6	+	770	0	0	0
30	7	++++	1,452	2	0	0
	8	++++	1,583	5	1	0
	9	++++	1,394	4	1	0
	10	++	611	1	0	0
	11	+	1,275	4	0	0
	12	+	920	0	1	0
45	13	+++	1,516	0	1	1
	14	+++	1,040	8	0	0
	15	++	1,009	6	1	0
	16	++	1,148	4	1	0
	17	+	554	0	1	0
60	19	+	1,024	6	2	0
	20	+	880	2	0	0
90	25	+	664	12	2	0

The overall severity of the myocarditis or myositis was defined according to the frequency of occurrence of histological patterns as follows: ++++ was assigned to tissues showing multiple necrotic foci converging to form single larger lesions; +++ was used when isolated necrotic foci were present but had not converged; ++ denoted the presence of inflammatory cell infiltrates but only occasional muscle fiber necrosis; + was used to refer to tissues with inflammatory cells but no necrotic development. When the tissues were essentially preserved and showed no significant accumulation of inflammatory cells, the assigned grade was −.

In each type of area we screened a total of 100–200 microscopic fields (×1,000) at separate lesion sites. The results were expressed as the average number of cells per 100 fields.

RESULTS

Table I describes the distribution of the PT, II, and DN types of lesions in the animal population during the acute and chronic stages of the infection. The most severe lesions (DN type) occurred in a relatively large proportion of animals during the early stages but were no longer seen after day 45 PI, i.e., during the chronic stage.

TABLE IV. *Type and distribution of inflammatory cells and amastigote nests in DN lesions of the heart.*

Day PI	Mouse no.	Severity of myocarditis	No. cells or amastigote nests per 100 fields: Mononuclear	Neutrophils	Eosinophils	Nests
15	1	+++	2,988	89	0	1
	2	+++	2,110	16	0	1
	3	++	1,225	1	1	0
30	7	++++	4,970	134	1	11
	8	++++	3,177	1,069	6	22
	9	++++	3,028	1,304	4	42
	10	++	1,017	2	1	0
45	13	+++	4,665	29	1	1
	14	+++	4,097	93	3	1
	15	++	2,468	17	0	0
	16	++	2,054	4	1	0

Findings in the myocardium of infected mice

In myocardial areas of the PT type, NEU and EOS levels were comparable to those of control mice but the numbers of MNC were systematically greater than the control values (Table II). There was no obvious correlation between the number of MNC and the overall severity of myocarditis, and amastigote nests were never found in PT areas. In myocardium sites displaying the histologic pattern II, MNC infiltration was readily detectable; the numbers of MNC were systematically greater than found in PT areas (Table III). At these sites, NEU or EOS infiltration was minimal and amastigote nests were rarely seen.

Mononuclear cells were the predominant inflammatory cells in the myocardial infiltrates of DN areas (Table IV). On day 30 PI, DN lesions were present in most mice and also contained relatively large numbers of NEU and amastigote nests. At this time, the mice presented the most severe necrotic and degenerative myocarditis.

Findings in skeletal muscle of infected mice

As in the myocardium, PT areas of skeletal muscle presented minimal, sometimes undetectable, NEU and EOS infiltration, comparable to control values (Table V). In these areas MNC always exceeded the control levels but by a relatively small margin. Amastigote nests were absent in PT areas. At lesion sites showing the II pattern, MNC infiltration was more pronounced than in PT areas and EOS infiltration became readily demonstrable in most mice (Table VI).

As in the myocardium, DN lesions were found in skeletal muscle only during the initial 45 days PI. In these lesions, MNC were again the predominant inflammatory cells (Table VII). On day 30 PI, amastigote nests were present in maximal numbers in DN areas and EOS accumulation was a typical feature of these lesion sites. The number of eosinophils per 100 fields systematically exceeded those found in areas exhibiting the less severe II or PT histologic patterns. It was also noted that, at any time during the initial 45 days PI, the numbers of EOS were much larger in DN areas of skeletal muscles than in DN areas of the myocardium (cf. Tables IV, VII).

TABLE V. *Type and distribution of inflammatory cells and amastigote nests in PT lesions of skeletal muscles.**

Day PI	Mouse no.	Severity of myositis	No. cells or amastigote nests per 100 fields: Mononuclear	Neutrophils	Eosinophils	Nests
15	1	++	150	3	2	0
	2	−	250	1	0	0
	3	+	168	1	0	0
	4	++	180	3	5	0
	5	+	126	0	0	0
	6	+	146	1	1	0
30	7	+++	299	1	3	0
	8	++++	283	9	2	0
	9	++++	292	2	2	0
	10	−	239	0	2	0
	11	−	229	0	1	0
	12	−	142	1	1	0
45	13	++++	230	9	6	0
	14	++++	258	0	2	0
	15	++++	195	2	5	0
	16	++++	246	0	0	0
	17	−	153	0	4	0
	18	−	130	0	0	0
60	19	+	172	0	1	0
	20	+	282	0	2	0
	21	−	260	1	1	0
	22	−	202	0	2	0
	23	−	168	1	2	0
	24	+	188	0	0	0
90	25	+	236	1	2	0
	26	−	204	0	1	0
	27	+	148	0	2	0
	28	−	186	0	1	0
	29	−	150	1	3	0
	30	−	142	0	5	0

* The average numbers of MNC, neutrophils, and eosinophils in skeletal muscles from uninfected age-matched mice did not vary significantly with age and were: 39 ± 7, 0.1 ± 0.2, and 0.9 ± 0.5, respectively.

DISCUSSION

These results defined the numerical composition of the inflammatory cell infiltrates of the heart and skeletal muscles of mice infected with *Trypanosoma cruzi* in relation to lesions of varying degrees of severity that develop during the acute and chronic stages of the disease. We also established the common and different charac-

TABLE VI. *Type and distribution of inflammatory cells and amastigote nests in II lesions of skeletal muscles.*

Day PI	Mouse no.	Severity of myositis	No. cells or amastigote nests per 100 fields			
			Mononuclear	Neutrophils	Eosinophils	Nests
15	1	+ +	948	11	4	9
	3	+	832	10	11	1
	4	+ +	950	8	2	1
	5	+	1,066	30	36	2
	6	+	1,184	26	12	2
30	7	+ + +	1,582	6	10	0
	8	+ + + +	1,477	87	4	2
	9	+ + + +	1,163	79	5	4
45	13	+ + + +	1,790	17	10	0
	14	+ + + +	1,742	8	36	0
	15	+ + + +	1,625	4	20	0
	16	+ + + +	2,056	14	20	0
60	19	+	947	0	0	0
	20	+	920	0	1	0
	24	+	835	1	1	0
90	25	+	859	2	1	0
	27	+	797	0	1	0

TABLE VII. *Type and distribution of inflammatory cells and amastigote nests in DN lesions of skeletal muscles.*

Day PI	Mouse no.	Severity of myositis	No. cells or amastigote nests per 100 fields			
			Mononuclear	Neutrophils	Eosinophils	Nests
15	1	+ +	2,642	157	25	10
	4	+ +	2,580	80	36	12
30	7	+ + +	4,441	18	57	9
	8	+ + + +	3,997	451	18	14
	9	+ + + +	3,794	561	22	17
45	13	+ + + +	5,162	182	110	0
	14	+ + + +	4,889	185	390	2
	15	+ + + +	4,052	23	185	0
	16	+ + + +	4,890	193	260	4

teristics of the inflammation of these 2 types of muscle tissues. In the myocardium, a correlation between relatively large numbers of MNC or NEU and abundant amastigote nests in DN areas was frequently observed on day 30 PI (Table IV). This gave an approximate idea of the time involved in the development of the most severe lesions and implied that parasites released from infected host cells and/or host cell debris could initiate the process that attracts inflammatory cells. The noted correlation was typically seen in mice presenting the most severe overall myocarditis, i.e., relatively large numbers of DN lesions.

In the myocardium, MNC were the first inflammatory cells to accumulate and their number increased with lesion severity (cf. Tables II, III, V). Neutrophils occurred in significant numbers in DN lesions but in minimal numbers in PT and II lesions. Conjecturally, MNC might mount immunologic responses against the parasite and NEU would be recruited thereafter to participate in the clearance of parasites (Villalta and Kierszenbaum, 1983) and tissue debris. Also, it is conceivable that NEU could contribute to lesion aggravation. It remains to be demonstrated whether tissue areas with the PT pattern become, in time, II lesions. If such were the case, our results would point to MNC infiltration as the first step in lesion development.

Mononuclear cells were also the first to accumulate in skeletal muscle lesions. Their number was linked to lesion severity and the overall degree of myositis. A comparison of the distribution of inflammatory cells in DN, II, and PT areas of skeletal muscles (cf. Tables V–VII) revealed that the numbers of all cell types increased with lesion severity. Hence, inflammatory cell numbers could be used to evaluate lesion severity in numerical terms, i.e., subject to statistical treatment. The consistently observed accumulation of EOS in areas II and DN of skeletal muscle was a most salient difference with the histology of comparable myocardial lesions (cf. Tables IV, VII). Whether lesion development in these 2 tissues follows different pathways is an intriguing possibility deserving attention.

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DEMONSTRATION OF AGGREGATION IN FREE-LIVING NYMPHS AND ADULTS OF THE BONT TICK, *AMBLYOMMA HEBRAEUM*

Trevor N. Petney
Tick Research Unit, Rhodes University, Grahamstown 6140, South Africa

ABSTRACT: Unfed males, females, and nymphs of *Amblyomma hebraeum* aggregated in petri dishes, as did combinations of any 2 of the above life history stages. Aggregations were not the result of thigmokinesis. Evidence is presented indicating that a pheromone is involved at least for nymphs. The primitive nature of aggregation in free-living ticks is discussed by comparison with the tick pheromone systems of other tick species.

Aggregation in free-living stages of ticks has been inferred from the nonrandom distribution of ticks on their hosts (Bull, 1978), from laboratory experiments (Browning, 1976; Treverrow et al., 1977; Petney and Bull, 1981), as well as from direct field observations (Browning, 1976; Sutherst et al., 1986). It has been found in species from many genera including both ixodid and argasid ticks (Leahy et al., 1975; Treverrow et al., 1977; Petney and Bull, 1981) and may occur in all life history stages (Leahy, 1979; Petney and Bull, 1981).

Aggregation results from pheromonal attraction in both argasid and ixodid ticks (Leahy, 1979; Sonenshine, 1985). In several cases the attraction has been shown to be active between species (Leahy et al., 1975; Petney and Bull, 1981). The commonness of aggregation behaviour, and of interspecific attraction to aggregations suggested that the behaviour of forming free-living aggregations is a primitive, ancestral feature of tick biology maintained by stabilizing selection (Petney and Bull, 1981).

Some members of the genus *Amblyomma* possess a unique pheromone system in which fed parasitic males attract males, females, and nymphs of the same species to attach nearby (Sonenshine, 1985). To date there is no information on whether these species form free-living aggregations. If, as hypothesized by Petney and Bull (1981), the nonparasitic aggregation is an ancestral behaviour maintained by stabilizing selection, then all groups of ticks should possess this behaviour, independent of variation on other communication systems. *Amblyomma hebraeum* is a tick in which attached males produce pheromones that attract other ticks (Rechav et al., 1977). This paper investigates whether this species also forms aggregations during its free-living phase.

In this paper I will use the term "free-living aggregations" to describe groupings of nonparasitic, unfed ticks and "attachment aggregations" for groupings of ticks around fed males.

MATERIALS AND METHODS

Free-living male, female, and nymphal *A. hebraeum* were obtained from the South African Bureau of Standards, East London. Prior to the commencement of experiments, the ticks were kept unfed in complete darkness at 85% relative humidity and 27 C. During experiments, the temperature was maintained at 27 C and relative humidity varied from 30 to 55%. All adults were between 2 wk and 3 mo postmoulting at the time of the experiment while the nymphs ranged from 1 to 3 wk postmoulting.

The tendency of free-living males, females, and nymphs to aggregate was tested by placing 10 individuals either of one life history stage or comprising 5 individuals of each possible combination of 2 life history stages in plastic petri dishes (8.8 cm diameter). These were left for 24 hr in the dark and the position of each individual was recorded thereafter. In addition, the number of individuals in physical contact with other individuals, either of the same (10 replicates each) or different life history stages (6 replicates each) was recorded. For single life history stages, Lloyd's index of patchiness (Southwood, 1978; Petney and Bull, 1981) was calculated after having divided the petri dish into 10 equal segments, thus giving a mean density of 1 tick per segment. Aggregated distributions could be statistically differentiated from random distributions when the mean value of Lloyd's index was greater than 1.

A test was made of the role of thigmokinesis in the formation of aggregations. Males, females, and nymphs, 100 each, were killed in alcohol then washed 3 times in distilled water. These were then glued either to the top (10 replicates for each life history stage) or bottom (10 replicates for each life history stage) of 8-cm diameter petri dishes, in a pentagonal pattern. After the glue had dried each petri dish was washed 3 times in distilled water, then dried at 27 C for 24 hr. Washing and drying were repeated each time the petri dish was reused. A single live tick corresponding to the life history stage in the petri dish was placed into the bottom center of each dish and left in the dark at 27 C and 85% relative humidity for 24 hr. At the end of this

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TABLE I. *The mean number of ticks (±SE) and percent of ticks in physical contact with other ticks are given for trials involving 2 different life history stages in the same petri dish.**

Mean number of	$\bar{x}$	SE	%
a) Males and females in the same petri dish:			
Males in contact only with other males	1.00	0.45	20.0
Females in contact only with other females	0.33	0.33	6.7
Males in contact with females	2.83	0.31	56.7
Females in contact with males	3.83	0.31	76.7
b) Males and nymphs in the same petri dish:			
Males in contact only with other males	0.33	0.33	6.7
Nymphs in contact only with other nymphs	0.00	—	0.0
Males in contact with nymphs	3.83	0.31	76.7
Nymphs in contact with males	3.00	0.36	60.0
c) Females and nymphs in the same petri dish:			
Females in contact only with other females	0.00	—	0.0
Nymphs in contact only with other nymphs	0.33	0.33	6.7
Females in contact with nymphs	4.16	0.31	83.3
Nymphs in contact with females	3.33	0.42	66.7

* Values are given for ticks contacting the same life history stage only and for those contacting the other life history stage. A total of 6 replicates each involving 5 individuals of each life history stage were carried out for each life history stage combination. Data are not arcsine-transformed as they are only descriptive.

time the position of each animal was recorded. A total of 50 replicates was run for each life history stage with dead ticks at the top and bottom of the petri dish.

Nymphs were used to test whether pheromones could induce aggregation. Ten squares of filter paper (1 cm^2) per vial were placed into each of 20 vials. In 10 vials the squares were left as controls; 10 nymphs were introduced into each of the other 10 vials to produce test squares. The 2 sets of vials were then placed into separate desiccators at 85% RH and 27 C and left in the dark for 2 wk. In each trial 1 nymph was placed into the center of a petri dish with a test and a control square on opposite sides of the dish (Petney and Bull, 1981). The side occupied by the test square varied between trials. Petri dishes were left in the dark for 24 hr and then the position of each tick recorded relative to the test and control squares. In total, 76% of the nymphs were in contact with the filter papers, which covered less than 4% of the available area.

RESULTS

In single life history stage trials a mean number (±SE) of 6.6 (0.41) males, 8.3 (0.50) females, and 6.7 (0.68) nymphs occurred in aggregations/petri dish. This is consistently over half the total of 10 individuals/petri dish. When 2 life history stages were present in the same petri dish most individuals were grouped in aggregations made up of both life history stages rather than in aggregations made up of individuals of only 1 life history stage (Table I). The aggregation between individuals was reflected by the high significance levels found for all life history stages using Lloyd's index (Table II). Comparisons between single life history stage trials show that there were no differences in the number of aggregations per petri dish nor in the number of individuals per aggregation (Table II).

In trials to test the response of live ticks to dead ticks it was found that 65% of males, 60% of females, and 64% of nymphs were found either on the top or bottom of the dish. The remaining animals were on the sides of the petri dish. All ticks were inactive at the time of recording. Very few of the live ticks were found in contact with the dead ticks (Table III). This is independent of life history stage or orientation of the dead ticks within the petri dish. Few ticks were found contacting corners between sides and top or bottom of the petri dish.

In the final experiment, 67 nymphs were found on the test filter paper squares while only 9 were found on the control squares giving a significant difference of $\chi^2_1 = 44.26$ ($P < 0.001$). The remaining 24 individuals were not in contact with either filter paper.

DISCUSSION

Males, females, and nymphs of *A. hebraeum* all showed a strong tendency to make contact with other individuals either of the same or a different life history stage. The lack of contact beteen live ticks and dead, washed ticks in their vicinity suggests that thigmokinesis is not a factor influencing free-living aggregations. Evidence from the experiment on nymphs indicates that at least this life history stage produces some chemical that is attractive to other nymphs. Although not demonstrated here, it is likely that

TABLE II. *The mean value for Lloyd's index of patchiness ($\bar{L}$) is given for trials involving single life history stages.**

[illegible]	Number of replicates	$\bar{L}$	t	P	No. aggregations/petri dish (SE)	No. ticks/aggregation (SE)
Mal[illegible]	[illegible]	2.74	2.98	<0.05	1.6 (0.16)	4.1 (0.59)
[illegible]	[illegible]	3.60	5.20	<0.001	1.9 (0.23)	4.2 (0.79)
[illegible]	[illegible]	[illegible]	4.58	<0.001	1.7 (0.26)	3.9 (0.73)

* This me[illegible] calculated for a random distribution ($\mu = 1$) using a t-test with 9 df. The t-value (t) and significan[illegible]s/petri dish (±SE) and the mean number of ticks/aggregation (±SE) are also given.

TABLE III. *The number of individuals (% in brackets) in each life history stage either in contact with or not in contact with dead, washed ticks placed in a petri dish are given. Dead ticks were placed either on the top or bottom of the petri dish.*

	In contact with tick (%)	Not in contact with tick (%)
Males		
Dead male tick at top of petri dish	0 (0.0)	50 (100.0)
Dead male tick at bottom of petri dish	0 (0.0)	50 (100.0)
Females		
Dead female tick at top of petri dish	0 (0.0)	50 (100.0)
Dead female tick at bottom of petri dish	1 (2.0)	49 (98.0)
Nymphs		
Dead nymphal tick at top of petri dish	2 (4.0)	48 (96.0)
Dead nymphal tick at bottom of petri dish	0 (0.0)	50 (100.0)

free-living aggregations of adults also have a pheromonal basis as such pheromones have regularly been found in other adult ticks (Graf, 1975; Treverrow et al., 1977).

The presence of free-living aggregations and a pheromonal component involved in such aggregations is not unexpected as each has frequently been found in other tick species (Sonenshine, 1985). It is of interest, however, that these experiments extend the result to a member of the genus *Amblyomma* in which aggregation/attachment pheromones, produced by fed males, are known to exist (Rechav et al., 1977). Free-living aggregations with a pheromonal basis are found in argasid ticks (Leahy et al., 1973, 1975), prostriate ticks (Graf, 1975; Sonenshine, 1985), and throughout the metastriate ticks, including both *Amblyomma* species that are known not to produce aggregation attachment pheromones (Treverrow et al., 1977; Petney and Bull, 1981) and *Amblyomma* species that do.

Neither the female-produced sex pheromone system nor the male-produced aggregation/attachment pheromone system is so uniformly found among ticks. Only *A. hebraeum* (Rechav et al., 1977), *A. maculatum* (Gladney et al., 1974a, 1974b), and *A. variegatum* (Norval and Rechav, 1979) are known to have the latter, while the former has not been found in the prostriate or in any apparently functional form in the *Amblyomma* species possessing aggregation/attachment pheromones (Sonenshine, 1985). This being so, it seems likely that the aggregation/attachment pheromones play a multiple role including the attraction of females to males, i.e., a sex pheromone component.

Mating behaviour and the role of pheromones in attracting sexes is thus varied within the ixodid ticks. The lack of variation within the free-living aggregation system conforms to the hypothesis put forward by Petney and Bull (1981) that this system is ancestral and subject to stabilizing selection.

Before a final analysis of the free-living aggregation in tick behaviour, 2 key factors remain to be elucidated. First, the chemical nature of the pheromone needs to be determined over a number of species to determine its conservatism. Second, the actual consequences of free-living aggregations, a point which is at present conjectural (Rechav and Whitehead, 1978; Leahy, 1979; Treverrow, 1980), remains to be determined.

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EFFECTS OF ALPHA- AND BETA-ADRENERGIC AGONISTS ON *TRYPANOSOMA CRUZI* INTERACTION WITH HOST CELLS

Mark C. Connelly*, Alfred Ayala, and Felipe Kierszenbaum†
Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101

ABSTRACT: We studied the effects of adrenergic agonists on the capacity of blood trypomastigote forms of *Trypanosoma cruzi* to associate with (i.e., bind and/or penetrate) host cells *in vitro*. The extent of *T. cruzi* association with mouse macrophages in the presence of the beta-adrenergic agonist L-isoproterenol was significantly decreased with respect to mock-treated controls. Similar results were obtained when the parasite was pretreated with L-isoproterenol and was then allowed to interact with untreated macrophages. In contrast, pretreatment of trypomastigotes with either L-phenylephrine or methoxamine—alpha-adrenergic agonists—enhanced their reactivity with macrophages. Interaction with a nonphagocytic host cell was also decreased and increased by parasite pretreatment with beta- and alpha-adrenergic agonists, respectively. The L-isoproterenol and L-phenylephrine effects were no longer detectable 2 and 3 hr after their removal, respectively, and were therefore reversible. Atenolol, a specific $beta_1$ adrenoreceptor blocker inhibited the L-isoproterenol effect, whereas butoxamine, a specific $beta_2$ blocker, did not. Thus, $beta_1$-like but not $beta_2$-like binding sites appeared to be expressed on *T. cruzi*. Both prazosin and yohimbine, preferential $alpha_1$- and $alpha_2$-receptor blockers, respectively, abolished the L-phenylephrine effect. The opposite effects of alpha- and beta-adrenergic agonists suggested that the infectivity of *T. cruzi* may be regulated by activation of surface components comparable to the adrenoreceptors. Trypomastigotes (66–79%) were found, by flow cytometry, to bind antibodies specific for the $beta_1$-adrenergic receptor of animal cells (turkey erythrocytes), suggesting a certain degree of similarity between *T. cruzi* and vertebrate cell $beta_1$ receptors. These results raise the possibility that natural alpha- and beta-adrenergic agonists may modulate *T. cruzi* infectivity *in vivo*.

The infective capacity of *Trypanosoma cruzi* trypomastigotes can be modified by cleaving surface sugar residues (Villalta and Kierszenbaum, 1983, 1984, 1985a, 1985b, 1987; Zenian and Kierszenbaum, 1983), protein moieties (Henriquez et al., 1981; Zenian and Kierszenbaum, 1983; Osuna et al., 1984), or phospholipid groups with enzymes (Connelly and Kierszenbaum, 1984, 1985), inhibiting metabolic pathways (Piras et al., 1982a; Osuna et al., 1984; Wirth and Kierszenbaum, 1985, 1986; Kierszenbaum et al., 1987), and altering the balance between intracellular levels of cyclic nucleotides (Wirth and Kierszenbaum, 1982, 1983, 1984a). Some of these modifications increase, whereas others decrease, the infectivity of the parasite, but all are reversible. The ability of the flagellate to recover from biochemical alterations that affect its ability to interact with host cells suggests that it is endowed with mechanisms that regulate its infectivity. A similar inference can be made from the gradual increase in the infective capacity ("maturation") that the trypomastigote form experiences shortly after being released from host cells (Piras et al., 1982b). However, our knowledge of these mechanisms is fragmented and limited. Our previous work on cyclic nucleotides and *T. cruzi* infectivity revealed that treatment with L-isoproterenol increases the level of adenosine 3′:5′-cyclic monophosphate (cAMP) in *T. cruzi* and decreases the extent of parasite association with murine macrophages (Wirth and Kierszenbaum, 1982). Because L-isoproterenol is a well-known beta-adrenergic agonist that acts on cells from vertebrates via activation of adenylate-cyclase-coupled beta-adrenoreceptors (reviewed by Exton, 1981, and by Lefkowitz et al., 1983), we decided to compare the effects of beta- and alpha-adrenergic agonists on the reactivity of *T. cruzi* with host cells and to study the effects of specific adrenoreceptor-blocking on parasite infectivity. It will be shown in this paper that alpha-adrenergic agonists enhance, whereas beta-adrenergic agonists inhibit, blood trypomastigote interaction with either phagocytic or nonphagocytic host cells *in vitro* and that these effects can be abrogated by agents that specifically block receptors for these agonists.

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* Present address: E. I. Du Pont de Nemours, Wilmington, Delaware 19898.
† To whom correspondence should be addressed.

MATERIALS AND METHODS

Parasites

The Tulahuén strain of *T. cruzi* was used in this work. Crl-CD-1(ICR)BR Swiss mice (Charles River Laboratory, Portage, Michigan) were infected by the intraperitoneal route with infected mouse blood containing 1×10^5 trypomastigotes. Ether-anesthetized

mice were exanguinated 2 wk later and the trypomastigotes were purified by centrifugation over Isolymph (Gallard-Schlesinger, Carle Place, New York) (Budzko and Kierszenbaum, 1974) followed by chromatography through a diethylaminoethyl-cellulose column (Mercado and Katusha, 1979). The recovered organisms were washed by centrifugation (800 *g*, 4 C, 15 min) using a phosphate-buffered saline solution containing 0.5% glucose and resuspended at the appropriate concentration in Dulbecco's modified minimal essential medium (Dutchland Laboratories, Denver, Colorado) containing 100 units/ml penicillin, 100 μg/ml streptomycin (DMEM), plus 1% bovine serum albumin (DMEM + BSA) (Sigma Chemical Co., St. Louis, Missouri). Parasite concentrations were determined microscopically using a hemacytometer.

Cell cultures

Monolayers of resident peritoneal macrophages from CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) or rat heart myoblasts (RHM) were prepared on the 3-mm-diameter glass wells of otherwise Teflon-coated microscope slides (Cel-Line, Newfield, New Jersey) as described in detail elsewhere (Lima and Kierszenbaum, 1982; Wirth and Kierszenbaum, 1984b). The cultures were incubated with DMEM supplemented with 10% heat-inactivated (56 C, 30 min) fetal bovine serum (Hyclone, Logan, Utah) at 37 C for 18 hr in a 5% CO_2-in-air moist atmosphere and washed with fresh DMEM immediately before use.

Reagents

L-isoproterenol, L-phenylephrine, methoxyamine, yohimbine hydrochloride, atenolol, and DL-propranolol were purchased from Sigma. Prasozin and butoxamine were gifts from Pfizer Laboratories (New York, New York) and Burroughs Wellcome Co. (Research Triangle Park, North Carolina), respectively. The solutions of these reagents used in the experiments were made in DMEM + BSA. At the tested concentrations and conditions, none of these reagents affected the viability or motility of the parasite.

Assay for host cell interaction with *T. cruzi*

The method for measuring *T. cruzi* association with host cells (i.e., surface binding and internalization) *in vitro* has been described (Wirth and Kierszenbaum, 1984b). Briefly, macrophage or RHM monolayers were prepared on 3-mm glass culture wells and incubated at 37 C (5% CO_2 incubator) for 2 hr with 15 μl of parasite suspension containing 1×10^6 organisms/ml. Under these conditions, the initial parasite:macrophage and parasite:RHM ratios were 5:1 and 10:1, respectively. The reagents tested were either present during the 2-hr parasite–macrophage interaction period or were used to pretreat the trypanosomes or the host cells. Unless otherwise noted, pretreatment of parasites or host cells with L-isoproterenol lasted 30 min, whereas pretreatment with L-phenylephrine or methoxamine lasted 60 min (37 C, 5% CO_2). Free reagent was removed by washing with DMEM by centrifugation at 800 *g* and 4 C for 15 min for the parasite, and rinsing for the host cell monolayers. Control parasite or host cell treatments were performed with medium alone. When agents known to block alpha- and beta-adrenergic agonists were to be tested, they were present in the same medium as the agonist. The cultures were terminated by washing with DMEM and fixation with absolute methanol. The slides were then stained with Giemsa and examined microscopically (×1,000). Not less than 200 microscopic fields were screened in each culture, recording the number of host cells with 1 or more or without parasites and the number of organisms associated with these cells. The results were expressed as the percentage of host cells with parasites and the average number of organisms per 100 host cells ± SD. All conditions were tested in triplicate.

Flow cytometry studies

Separate 25-μl aliquots of suspensions of purified trypomastigotes (at 4–8 × 10^7 organisms/ml) in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (diluent) were incubated in an ice bath for 1 hr with an equal volume of rabbit antiserum specific for the 43,000-Dalton form of the turkey erythrocyte beta$_1$-adrenergic receptor (anti-BAR_{43}), rabbit antiserum specific for a peptide fragment including amino acids in positions 146–161 of the turkey erythrocyte beta$_1$-adrenergic receptor (anti-BAR_1), and normal rabbit serum. These sera were a generous gift from Dr. M. D. Minnich (Smith Kline and French, Philadelphia, Pennsylvania); the details of their preparations have been published (Shorr et al., 1987). At the end of the incubation period the volume of the mixtures was brought up to 1 ml with diluent and they were centrifuged (800 *g*, 4 C, 15 min). The pelleted organisms were resuspended in 25 μl of a 1/10 dilution (in diluent) of fluorescein-conjugated $F(ab')_2$ from goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pennsylvania) and were incubated in an ice bath for 1 hr. After removal of the excess $F(ab')_2$ reagent and washing by centrifugation, the flagellates were resuspended in diluent containing 1% formaldehyde for determination of the proportion of fluorescent organisms in an Ortho Cytofluorograph 50-H interfaced with a model 2150 computer (Ortho Diagnostics, Westwood, Connecticut). A minimum of 10,000 trypomastigotes was examined in each sample.

Presentation of data and statistical analysis

The sets of results presented in the tables of this paper are typically representative of 2–4 separate repeat experiments for each protocol. Differences were considered to be significant if $P < 0.05$, determined by the Mann-Whitney *U*-test.

RESULTS

Effects of alpha- and beta-adrenergic agonists on *T. cruzi* interaction with mammalian host cells

When trypomastigotes were cocultured with macrophages in the presence of various concentrations of the beta-adrenergic agonist L-isoproterenol, both the percentage of cells with parasites and the average number of organisms per 100 cells were significantly decreased with respect to the values obtained with mock-treated cultures (Table I). Although 1×10^{-7} M L-iso-

TABLE I. *Effects of L-isoproterenol, L-phenylephrine, and methoxyamine on* T. cruzi*–macrophage interaction.**

Agent	Molarity	% Macrophages with parasites	Number of parasites per 100 macrophages†
L-isoproterenol	0	14.1 ± 0.4	16.1 ± 0.5
	1×10^{-7}	13.0 ± 3.2	14.6 ± 2.6
	1×10^{-6}	9.0 ± 1.1 (−36)	9.7 ± 1.5 (−40)
	1×10^{-5}	7.1 ± 1.1 (−50)	7.3 ± 1.4 (−55)
	1×10^{-4}	7.5 ± 2.5 (−47)	7.7 ± 2.8 (−52)
L-phenylephrine	0	16.0 ± 0.8	18.1 ± 1.5
	1×10^{-9}	23.1 ± 4.8 (44)	27.8 ± 6.1 (54)
	1×10^{-8}	29.1 ± 2.7 (82)	37.5 ± 6.8 (107)
Methoxyamine	0	36.8 ± 5.7	53.4 ± 8.6
	1×10^{-9}	45.7 ± 2.8 (24)	77.6 ± 5.4 (45)
	1×10^{-8}	49.6 ± 4.2 (35)	77.4 ± 7.9 (45)

* Macrophages and parasites were cocultured at 37 C in the absence or presence of the indicated reagent. After removal of the free parasites, the cultures were terminated by fixation and stained. The experiments with each adrenergic agonist were performed separately. In this and subsequent tables the results represent the mean ± SD, and the difference between the experimental and the corresponding control value is expressed as a percentage of the control and given in parentheses where statistically significant ($P < 0.05$).

† Bound and/or internalized parasites.

proterenol did not cause a significant effect in the experiment represented in Table I, reduced interaction was seen in some of the repeat experiments (data not shown). Both alpha-adrenergic agonists, L-epinephrine or methoxyamine, markedly enhanced parasite–macrophage interaction.

To find out which of the interacting cells was affected by the tested agonists, we separately pretreated each cell and then cocultured it with the untreated counterpart. As shown in Table II, macrophage pretreatment with 1×10^{-5} M or 1×10^{-4} M, but not with 1×10^{-6} M, L-isoproterenol resulted in reduced association with untreated *T. cruzi*. Instead, pretreatment with concentrations of L-phenylephrine and methoxyamine that had altered macrophage–parasite interaction when continuously present in the cocultures had no effect on the macrophages.

When the parasites were pretreated with L-isoproterenol, L-phenylephrine, or methoxyamine, the direction of ensuing changes was the same as observed when the reagents had been continuously present during the interaction (cf. Tables I, III). Given the similar effects of L-phenylephrine and methoxyamine, we used only L-phenylephrine in subsequent experiments.

To determine the minimal parasite pretreatment time to cause the L-isoproterenol and L-phenylephrine effects we incubated *T. cruzi* with these agents for various periods of time and washed them before their addition to untreated macrophage culture. The L-isoproterenol effect was detectable after 5 min, i.e., when first tested, and remained demonstrable after 30 min (Table IV). The L-phenylephrine was detectable after 15 min but not after 5 min.

We also looked into whether or not the L-isoproterenol and L-phenylephrine effects were reversible. In this case, the flagellates were treated with the appropriate agonist, washed, and were then incubated with fresh medium for various periods of time before their addition to macrophage cultures. As shown in Table V, the L-isoproterenol and L-phenylephrine effects became undetectable 2 and 3 hr, respectively, after removal of the free agonist. In these experiments,

TABLE II. *Effects of pretreatment of macrophages with adrenergic agonists on their interaction with* T. cruzi.*

Exp. no.	Agent	Molarity	% Macrophages with parasites	Number of parasites per 100 macrophages
1	L-isoproterenol	0	14.6 ± 3.4	16.5 ± 4.4
		1×10^{-6}	11.3 ± 3.2	12.2 ± 1.9
		1×10^{-5}	6.1 ± 1.1 (−58)	6.5 ± 1.2 (−61)
		1×10^{-4}	4.0 ± 0.3 (−73)	4.0 ± 0.3 (−76)
2	L-phenylephrine	0	11.4 ± 2.5	12.9 ± 3.2
		1×10^{-9}	12.9 ± 1.7	14.8 ± 2.0
		5×10^{-9}	12.8 ± 2.5	13.5 ± 2.5
		1×10^{-8}	11.3 ± 1.3	12.1 ± 1.5
	Methoxyamine	1×10^{-9}	9.4 ± 0.3	10.3 ± 0.7
		5×10^{-9}	9.6 ± 1.5	10.5 ± 1.8
		1×10^{-8}	10.0 ± 1.7	10.6 ± 1.7
		5×10^{-8}	10.0 ± 3.1	11.3 ± 4.0

* Macrophages were incubated with medium alone or containing the indicated concentration of L-isoproterenol (37 C, 30 min), L-phenylephrine (37 C, 60 min), or methoxyamine (37 C, 60 min) and washed prior to incubation with untreated parasites.

TABLE III. *Effects of pretreatment of* T. cruzi *with adrenergic agonists on its interaction with macrophages.**

Exp. no.	Agent	Molarity	% Macrophages with parasites	Number of parasites per 100 macrophages
1	L-isoproterenol	0	15.0 ± 1.5	16.4 ± 2.7
		1×10^{-7}	6.5 ± 2.3 (−57)	9.1 ± 1.8 (−45)
		1×10^{-6}	7.6 ± 1.6 (−49)	8.0 ± 1.6 (−51)
		1×10^{-5}	6.4 ± 2.8 (−57)	6.9 ± 3.5 (−58)
2	L-phenylephrine	0	16.0 ± 1.3	17.5 ± 1.4
		1×10^{-9}	21.6 ± 3.0 (32)	25.4 ± 3.4 (45)
		5×10^{-9}	29.6 ± 1.6 (85)	36.9 ± 3.5 (111)
		1×10^{-8}	25.2 ± 3.9 (58)	29.0 ± 3.7 (66)
	Methoxyamine	1×10^{-9}	19.4 ± 0.4 (21)	22.8 ± 1.6 (30)
		5×10^{-9}	26.5 ± 3.5 (66)	34.1 ± 7.8 (95)
		1×10^{-8}	25.6 ± 2.0 (60)	31.4 ± 3.0 (79)
		5×10^{-8}	26.0 ± 2.4 (63)	30.8 ± 3.2 (82)

* Parasites were incubated with medium alone or containing L-isoproterenol (37 C, 30 min), L-phenylephrine (37 C, 60 min), or methoxyamine (37 C, 60 min), and washed prior to being added to macrophage cultures.

controls were included for each time period to account for any time-dependent variation in parasite infectivity that might have occurred.

The above results could be explained in terms of an altered capacity of the trypanosome to bind or invade macrophages, and with the possibility that the agonists induced changes in the parasite affecting its susceptibility to being phagocytosed. To explore these possibilities, we used as host cells RHM which, not being phagocytic, can only be infected by cell membrane penetration. Pretreatment of *T. cruzi* with L-isoproterenol or L-phenylephrine decreased and increased, respectively, its capacity to interact with RHM (Table VI).

TABLE IV. *Effects of parasite pretreatment with adrenergic agonists for varying periods of time on its interaction with macrophages.**

Agent tested	Incubation time (min)	% Macrophages with parasites	Number of parasites per 100 macrophages
L-isoproterenol	0	12.5 ± 1.6	15.1 ± 1.9
	5	5.6 ± 1.3 (−55)	5.8 ± 1.0 (−62)
	15	7.4 ± 2.7 (−41)	7.7 ± 2.4 (−49)
	30	7.8 ± 1.9 (−57)	9.1 ± 1.3 (−40)
	60	12.0 ± 0.7	13.4 ± 0.9
	90	12.3 ± 3.2	15.1 ± 2.9
L-phenylephrine	0	17.3 ± 6.4	20.2 ± 7.8
	5	23.7 ± 6.5	30.6 ± 7.8
	15	26.0 ± 1.4 (50)	45.1 ± 4.5 (123)
	30	35.1 ± 4.4 (103)	46.1 ± 9.6 (128)

* The parasites were incubated with medium alone or containing 1 × 10 [illegible] M L-isoproterenol or 1×10^{-8} M L-phenylephrine for the indicated amounts of time, washed, and then added to untreated macrophage cultures.

Inhibition of the L-isoproterenol and L-phenylephrine effects by specific blockers of alpha- and beta-adrenergic receptors

If the receptors for alpha- and beta-adrenergic agonists on *T. cruzi* were similar to those found in vertebrate cells (Exton, 1981; Lefkowitz et al., 1983), agents that specifically block these receptors on the latter should act similarly on the parasite. Indeed, the L-isoproterenol effect on trypomastigote interaction with macrophages was abrogated by propranolol (a beta blocker that does not distinguish between the beta$_1$- and beta$_2$-adrenergic receptor subtypes), atenolol (a specific beta$_1$ blocker), but not by butoxamine (a specific beta$_2$ blocker) (Table VII). Moreover, the L-phenylephrine effect was abolished by either prazosin (which binds alpha$_1$-adrenergic receptors with much greater affinity than alpha$_2$ receptors) or yohimbine (which has greater affinity for alpha$_2$ receptors) (Exton, 1981; Lefkowitz et al., 1983) (Table VIII).

Flow cytometry studies

In two repeat experiments, the proportions of trypomastigotes displaying fluorescence after treatment with anti-BAR$_{43}$ were 73.9 and 79.3%, whereas the values obtained by using anti-BAR$_1$ were 73.4 and 66.3%. Only 6.4 and 6.7%, respectively, of the organisms were positive after treatment with normal rabbit serum, all other conditions remaining unchanged.

DISCUSSION

These results show that alpha- and beta-adrenergic agonists can induce significant changes in the capacity of *T. cruzi* to interact with mam-

TABLE V. *Reversibility of the effects of L-isoproterenol or L-phenylephrine on* T. cruzi.*

Exp. no.	Parasite treatment	Posttreatment time (hr)	% Macrophages with parasites	Number of parasites per 100 macrophages
1	DMEM + BSA	1	13.5 ± 2.5	14.5 ± 3.5
	L-isoproterenol	1	4.8 ± 1.9 (−64)	5.0 ± 2.1 (−66)
	DMEM + BSA	2	24.5 ± 4.7	33.4 ± 8.2
	L-isoproterenol	2	20.8 ± 3.6	28.7 ± 3.8
2	DMEM + BSA	1	24.9 ± 2.2	27.8 ± 2.3
	L-phenylephrine	1	40.0 ± 9.2 (61)	47.1 ± 11.5 (69)
	DMEM + BSA	2	26.9 ± 3.5	31.4 ± 6.2
	L-phenylephrine	2	37.3 ± 5.9 (39)	44.4 ± 6.7 (41)
	DMEM + BSA	3	23.4 ± 5.0	25.9 ± 5.9
	L-phenylephrine	3	28.3 ± 3.2	33.1 ± 4.4

* The parasites were incubated with medium alone or containing 1×10^{-5} M L-isoproterenol (37 C, 30 min) or 1×10^{-6} M L-phenylephrine (37 C, 60 min), washed, and incubated in fresh medium for the indicated periods of time prior to being added to untreated macrophage cultures.

malian host cells. That such capacity was being modulated rather than the susceptibility of the flagellates to being phagocytosed was indicated by the fact that L-phenylephrine caused the same type of effect whether phagocytic or nonphagocytic host cells were used. The opposite direction of the changes induced by alpha- and beta-adrenergic agonists suggested that these agents could influence a regulatory mechanism impinging on parasite association with (i.e., binding and penetration of) mammalian host cells.

L-isoproterenol increases intracellular levels of cAMP in macrophages (Wirth and Kierszenbaum, 1982), and this increase is associated with a reduced capacity of these cells to bind and take up blood forms of *T. cruzi* (Wirth and Kierszenbaum, 1982, 1983). The fact that 1×10^{-6} M (some cases 1×10^{-7} M) L-isoproterenol reduced the extent of parasite–macrophage interaction when it was present in the cocultures but not when it was used to pretreat the macrophage suggested that the contribution of the macrophage was not required for the inhibitory effect to occur. Instead, the enhanced *T. cruzi*–macrophage association induced by the alpha-adrenergic agonists appeared to be due exclusively to an effect on the trypanosomes because it was seen only after parasite pretreatment with either L-phenylephrine or methoxamine.

The maximal trypanosome pretreatment periods resulting in significant changes in parasite–host cell interaction were 5 min for L-isoproterenol and 6–15 min for L-phenylephrine. These observations defined the minimum time periods between the—presumable—activation of adrenergic receptors and the manifestation of consequences. The effects were reversible because they subsided and became undetectable within 2 and 3 hr for L-isoproterenol and L-phenylephrine, respectively. The ability of the parasite to recover suggested the existence in *T. cruzi* of a mechanism that regulates infectivity and can be modulated through activation of receptors comparable, at least in terms of affinity for the appropriate agonists and blockers, to alpha- and beta-adrenergic receptors. That either propranolol or atenolol, but not butoxamine, abrogated the inhibitory effect of L-isoproterenol suggested the presence of at least beta$_1$-like adrenergic receptors on *T. cruzi* trypomastigotes. The fact that relatively high proportions of trypomastigotes bound anti-BAR$_{43}$ and anti-BAR$_1$ antibodies, either one specific for the turkey erythrocyte beta$_1$-adrenergic receptor (Shorr et al., 1987), supported further this inference and indicated a certain degree of similarity between these receptors in *T. cruzi* and cells of vertebrates.

The presence of both alpha$_1$-like and alpha$_2$-like adrenergic receptors on the parasite was suggested by the abolishment of the stimulatory effect of L-phenylephrine by prazosin and yohimbine, respectively. Prazosin and yohimbine inhibited the L-phenylephrine effect at concen-

TABLE VI. *Effects of pretreatment of* T. cruzi *with L-isoproterenol or L-phenylephrine on its interaction with RHM.**

Parasite treatment	% RHM with parasites	Number of parasites per 100 RHM
DMEM + BSA	4.3 ± 1.0	4.4 ± 1.2
L-isoproterenol	1.5 ± 1.0 (−65)	1.5 ± 1.0 (−65)
L-phenylephrine	8.8 ± 1.6 (104)	9.0 ± 1.6 (105)

* The parasites were incubated with medium alone or containing 1×10^{-5} M L-isoproterenol or 1×10^{-6} M L-phenylephrine, and washed prior to being incubated with untreated macrophage cultures.

TABLE VII. *Effects of parasite pretreatment with L-isoproterenol in the absence or presence of a nonspecific beta (propranolol), beta$_1$ (atenolol), or beta$_2$ (butoxamine) blocker.**

Exp. no.	Parasite pretreatment	% Macrophages with parasites	Number of parasites per 100 macrophages
1	DMEM + BSA	11.3 ± 2.0	12.9 ± 3.1
	10^{-7} M L-isoproterenol	5.3 ± 1.5 (−53)	6.3 ± 1.8 (−51)
	10^{-5} M propranolol	10.7 ± 0.6	11.3 ± 0.3
	10^{-7} M L-isoproterenol + 10^{-6} M propranolol	6.8 ± 1.7 (−40)	7.0 ± 1.9 (−46)
	10^{-7} M L-isoproterenol + 10^{-5} M propranolol	9.5 ± 2.7	10.1 ± 2.6
2	DMEM + BSA	12.4 ± 3.3	13.5 ± 3.5
	10^{-7} M L-isoproterenol	4.6 ± 0.6 (−62)	4.8 ± 0.7 (−64)
	10^{-5} M atenolol	8.9 ± 1.7	10.1 ± 2.0
	10^{-5} M butoxamine	10.8 ± 0.8	11.6 ± 1.3
	10^{-7} M L-isoproterenol + 5 × 10^{-7} M atenolol	6.9 ± 1.6 (−44)	7.0 ± 1.8 (−48)
	10^{-7} M L-isoproterenol + 1 × 10^{-6} M atenolol	10.1 ± 3.1	11.1 ± 3.2
	10^{-7} M L-isoproterenol + 1 × 10^{-5} M atenolol	10.8 ± 1.6	10.9 ± 1.8
	10^{-7} M L-isoproterenol + 5 × 10^{-7} M butoxamine	6.1 ± 2.9 (−51)	6.3 ± 3.1 (−53)
	10^{-7} M L-isoproterenol + 1 × 10^{-6} M butoxamine	5.8 ± 1.2 (−53)	6.0 ± 1.1 (−56)
	10^{-7} M L-isoproterenol + 1 × 10^{-5} M butoxamine	5.0 ± 0.4 (−60)	5.7 ± 0.7 (−58)

* The parasites were incubated with medium alone or containing L-isoproterenol with or without the tested beta blocker (37 C, 2 hr), washed, and then allowed to interact with macrophages.

trations that were comparable to those used by other investigators to selectively block alpha$_1$ and alpha$_2$ receptors, respectively, on liver cell membranes (El-Refai and Exton, 1980; Jard et al., 1981). Therefore, it would seem unlikely that these blockers would affect more than 1 subtype of alpha receptor on the parasite. Nevertheless, a firm conclusion with respect to the existence of both subtypes of alpha receptors rather than a single subtype would require knowledge of the actual numbers of each receptor subtype on the trypomastigote. It should be noted, however, that although we cannot be certain at this time that both alpha-adrenergic receptor subtypes are expressed on *T. cruzi,* our results support the existence of at least one of them (or a structure of similar function) on *T. cruzi.*

Whereas activation of alpha$_1$ receptors in many tissues causes marked changes in cell calcium ion flux, activation of alpha$_2$ receptors leads to effects that are believed to result from decreases in intracellular cAMP levels (Exton, 1981). In almost all cases, the effects of beta-adrenergic receptor activation are mediated by the rise in cAMP that owed to stimulation of the membrane-bound enzyme complex adenylate cyclase (Lefkowitz et al., 1983). Adenylate cyclase has been identified in *T. cruzi* epimastigotes as well as in cultured trypomastigotes (Da Silveira et al., 1977; Rangel-Aldao et al., 1987), and cAMP has been postulated to play a role in the differentiation of *T. cruzi* (Rangel-Aldao et al., 1987). Furthermore, elevation of cAMP levels in blood forms of *T. cruzi* is accompanied by a significant decrease in

TABLE VIII. *Effects of parasite pretreatment with L-phenylephrine in the absence or presence of an alpha$_1$ (prazosin) or an alpha$_2$ (yohimbine) blocker.**

Parasite pretreatment	% Macrophages with parasites	Number of parasites per 100 macrophages
DMEM + BSA	12.2 ± 0.5	15.6 ± 0.9
10^{-8} M L-phenylephrine	24.7 ± 5.6 (102)	31.8 ± 7.4 (104)
10^{-5} M prazosin	13.2 ± 1.1	14.4 ± 1.3
10^{-5} M yohimbine	10.7 ± 0.5	12.1 ± 2.2
10 M L-phenylephrine + 5 × 10^{-8} M prazosin	8.8 ± 2.6	9.7 ± 3.3
10 M L-phenylephrine + 1 × 10^{-7} M prazosin	13.1 ± 2.9	15.0 ± 4.5
10 M L-phenylephrine + 5 × 10^{-6} M prazosin	11.7 ± 2.4	13.8 ± 2.9
10 M L-phenylephrine + 1 × 10^{-5} M prazosin	9.7 ± 2.5	10.9 ± 2.0
10 M L-phenylephrine + 5 × 10^{-8} M yohimbine	22.2 ± 2.5 (84)	26.0 ± 3.9 (67)
10 M L-phenylephrine + 1 × 10^{-7} M yohimbine	14.6 ± 3.1	16.1 ± 3.8
10 M L-phenylephrine + 1 × 10^{-6} M yohimbine	15.4 ± 3.0	16.7 ± 2.9
10 M L-phenylephrine + 1 × 10^{-5} M yohimbine	13.3 ± 2.0	17.1 ± 1.6

* The parasites were incubated wi[illegible] containing L-phenylephrine with or without thhe tested alpha blocker (37 C, 2 hr), washed, and then allowed to interact with macrophages.

parasite–macrophage association (Wirth and Kierszenbaum, 1982). This background information makes it tempting to speculate that some of the mechanisms set in motion in vertebrate cells after activation of alpha- and beta-adrenergic receptors may be involved in the regulation or maturation of the infective capacity of *T. cruzi.* Regardless of the biochemical events underlying the effects of the alpha- and beta-adrenergic agonists, the present results raise the possibility that adrenergic agonists, which naturally occur in body fluids, modulate parasite infectivity *in vivo.*

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CRYOPRESERVATION OF FIRST-STAGE AND INFECTIVE THIRD-STAGE LARVAE OF *STRONGYLOIDES STERCORALIS*

Thomas J. Nolan, Linda M. Aikens, and Gerhard A. Schad

University of Pennsylvania School of Veterinary Medicine, Department of Pathobiology, 3800 Spruce St., Philadelphia, Pennsylvania 19104-6050

ABSTRACT: Infective third-stage larvae of *Strongyloides stercoralis* were frozen over liquid nitrogen and remained infective to dogs when thawed. Successful cryopreservation depended on a 30–60-min incubation in a cryoprotectant (10% DMSO and 10% dextran) before freezing and thawing the frozen larvae into RPMI. First-stage larvae could also be frozen by this method. Thawed first-stage larvae remained viable and continued their development to third-stage larvae, which were shown to be infective to dogs.

Primates (Genta et al., 1984; Harper et al., 1984) and dogs (Grove and Northern, 1982; Schad et al., 1984) are the only established laboratory hosts for *Strongyloides stercoralis*. This narrow host range, involving relatively large hosts, makes the routine laboratory maintenance of this parasite expensive. Although previous studies have shown that some species of *Strongyloides* can be cryopreserved and viable larvae were obtained upon thawing, none of these earlier attempts produced larvae that were infective (Weinman and McAllister, 1947; Van Wyk et al., 1977). Thus, to date, there have been no reports of successful freezing of infective larvae for the routine maintenance of this genus (Neva, 1986).

Many parasitic nematodes, including *Haemonchus contortus* (Campbell et al., 1973), *Ancylostoma caninum* (Kelly et al., 1976), and several of the common nematodes of ruminants (Van Wyk et al., 1977), have been frozen and have retained their infectivity. The present study was initiated to develop a method for preserving the infectivity of frozen *Strongyloides stercoralis* larvae. Successful cryopreservation would allow us to maintain several isolates of the parasite, including our standard laboratory strain, without the extra expenses incurred through routine maintenance in laboratory animals.

MATERIALS AND METHODS

Strongyloides stercoralis

Strongyloides stercoralis was isolated from a naturally infected dog in 1980, and has been continuously maintained in puppies and immunosuppressed dogs (Schad et al., 1984). First-stage larvae (L1) were obtained by Baermannization of fresh feces from an infected dog. Third-stage larvae (L3) were reared in charcoal–feces cultures and were recovered after 7–10 days by Baermannization.

Freezing techniques

Larvae were washed twice in water and resuspended in 1 ml of freezing medium, containing cryoprotectants, in a plastic cryotube (NUNC, Denmark). The known cryoprotectants, dimethyl sulfoxide (DMSO), glycerol, and dextran (MW 9,400, 17,200, and 255,000) (Sigma Chemical Co., St. Louis, Missouri) were tested, either singly or in combination, for their ability to enhance survival. Using a 30-min incubation in the cryoprotectant solution as a standard, worms were frozen in a 10% (v/v), or in the case of the dextrans a 10% (w/v), concentration of the cryoprotectant in distilled water. After the optimal cryoprotectant was determined, the effect of incubation was tested by holding the worms for various times, up to 90 min, at room temperature before they were frozen in the vapor phase of liquid nitrogen (fast freezing) in a cryogenic storage tank (Union Carbide, Indianapolis, Indiana). Alternatively, after incubation, the worms were placed into a controlled rate freezing apparatus (Cryomed Model 1010, Cryomed, Mt. Clemens, Michigan) and the temperature (as measured in the vial) was lowered at the rate of 0.8 C/min to −40 C and then by 10 C/min until −70 C was reached (slow freezing), at which time the vials of frozen larvae were placed in the vapor phase of liquid nitrogen for long-term storage.

Vials of frozen larvae were prepared for thawing by adding 0.5 ml of cold (4 C) RPMI (GIBCO, Long Island, New York), water, or phosphate-buffered saline (PBS) to the frozen pellet. They were then held at 34–37 C, either in a water bath or in the palm of the hand, with occasional gentle inverting until thawed (approximately 3 min). The larvae were then resuspended in 10 ml of medium and centrifuged at 150 *g* for 7 min, and, finally, resuspended in 3 ml of fresh medium.

Viability determination

Thawed larvae were placed in 1 ml of RPMI in a 24-well tissue culture plate (Becton Dickinson Labware, Oxnard, California). After 20 min, the larvae were examined for motility using an inverted microscope and the percentage of motile larvae was determined. Obviously damaged worms (a ruptured cuticle with a protruding esophagus was the most common visible damage), even if motile, were counted as non-

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viable. In some cases, penicillin–streptomycin (100 U/ml, 100 μg/ml, respectively) was added to the wells and the worms were incubated at 26 C overnight and a second count was made.

Thawed L1's were also tested for their ability to develop either to L3's or to complete their heterogonic cycle. To test their developmental ability, the thawed larvae were placed on 1.5% agarose plates (FMC, Rockland, Maine), to the surface of which 5 μg of cholesterol in 1 ml ethanol had been applied, and given *E. coli* as a food source. Plates were incubated at 30 C and examined daily to qualitatively monitor larval development (no attempt was made to determine how many larvae actually developed into adults).

Infectivity determination

One-week-old puppies of mixed breeding were obtained from the Buckshire Corp. (Perkasie, Pennsylvania). They were examined for the presence of helminth eggs or larvae in their feces upon arrival and were determined to be helminth free. Thawed L3's were injected subcutaneously within 1 hr after thawing, into the flank of experimental dogs. Thawed L1's were grown on agar plates for 2 days to allow time for development to L3's, which were then collected and injected subcutaneously into dogs. Feces were collected starting at day 10 after inoculation and examined for larvae by Baermannization. When larvae were found in the fecal samples for 2 consecutive days, most dogs were treated with ivermectin (200 μg/kg per os) (Merck Sharp and Dohme, Rahway, New Jersey) and the experiment was terminated. The infection in 1 positive dog was allowed to continue untreated to determine if the output of larvae would reach levels high enough to insure that sufficient infective larvae could be obtained to infect another dog. This dog was immunosuppressed with corticosteroids (Schad et al., 1984) at 5 wk of age and was observed until high levels (>1,000 larvae/g) were being passed, when it too was treated with ivermectin and removed from the study. L1's collected from this last dog were grown in charcoal–feces cultures or on agar plates, to determine whether they would undergo heterogonic development.

RESULTS

Effect of cryoprotectant on larval survival

When L3's were frozen in water or PBS, none survived, whereas, in the presence of cryoprotectants and a 30-min incubation, larvae did survive. DMSO alone provided more protection (36 ± 11.3% motile at 20 min postthawing) than either low molecular weight (MW 9,400) dextran (19.5 ± 3.5%) or glycerol (2.5 ± 0.7%). The protection afforded by dextran was additive when it was used with either DMSO or glycerol (41.7 ± 12% and 22.3 ± 16.2%, respectively). Dextrans of higher molecular weights, when in combination with DMSO, gave varying results: MW 17,200 dextran gave the same degree of protection as the MW 9,400; however, MW 255,000 dextran gave a much lower degree of protection (14.4% survival vs. 61.9% for the lower weight dextrans with a 60-min incubation).

In order to determine the optimum concentration of cryoprotectant, the amount of DMSO was varied while holding the dextran constant at 10%. When the concentration of DMSO ranged from 9 to 11% (11% was the highest tested) there was little difference in worm survival. As the percentage decreased below 9% (0, 5, and 7% were tested), the number of survivors gradually decreased.

Effect of incubation in cryoprotectant before freezing

When L3's were placed in the combination of 10% DMSO (v/v) and 10% dextran (w/v) in water (1 ml DMSO + 1 g dextran + 8 ml water = 10% D+d) and immediately placed over liquid nitrogen, none of the larvae survived. When the incubation time before freezing was lengthened, increasing numbers of worms survived, until at 90 min 73.5 ± 9.2% survived (Fig. 1). L3's frozen in the combination of 10% glycerol and 10% dextran showed an increased survival varying directly with incubation time up to 30 min. Beyond this time there was no increase in survival, with about 20% of the larvae surviving after incubation for either 30 min or 60 min. After 30 min, larvae incubated in either cryoprotectant combination appeared crenated.

L1's needed less incubation in 10% D+d than L3's. In fact, 42.5 ± 13.7% survived without any incubation and 84.7 ± 5.5% survived after a 30-min incubation. Prolonging the incubation time to 60 min did not increase the survival of first-stage larvae (69.8 ± 23.8% survival).

Effect of freezing rate

Because the rate of freezing has an effect on the survival of cryopreserved organisms (James, 1985), we compared effect of fast freezing (placing the vial directly over liquid nitrogen) and slow freezing (where the rate of the temperature decrease was kept constant at 0.8 C/min by a controlled rate freezing apparatus) on larval survival. The freezing rate had no effect on the survival of L3's; 61.9 ± 8.0% were motile at 20 min postthawing when fast freezing was used, whereas 65.5 ± 10.9% survived slow freezing. L1's, however, survived slow freezing better than fast freezing (95.4 ± 6.1% vs. 56.8 ± 26.3%, respectively). A 60-min incubation and 10% D+d was used in all cases.

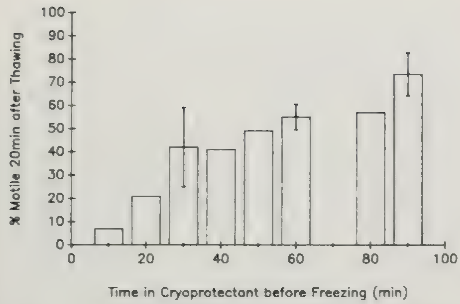

FIGURE 1. Effect of incubation in cryoprotectant before fast freezing L3's. Combined results of 5 experiments. Standard deviations are shown only for those time points that were included in more than 1 experiment. The cryoprotectant used was 10% DMSO and 10% dextran.

First-stage larvae vs. third-stage larvae

Thawed L1's, grown on agarose plates, developed to the adult stage, and, subsequently these gave rise to third-stage larvae. The time required for this developmental process was the same whether the agarose plates were inoculated with fresh or frozen L1's. On agarose plates, approximately 100% of the surviving L1's were alive and developing at 24 hr postthawing. In RPMI, between 21 and 79% of the surviving L1's were alive at 24 hr (the higher percentages were found in those larvae that had been slow frozen), whereas only 0.6–39% of L3's survived 24 hr, with no difference between fast and slow freezing.

The number of larvae frozen in a 1-ml vial did not seem to affect the survival of the worms; as few as 35/ml and as many as 50,000/ml were frozen with the same levels of survival (65.3 vs. 57.3% survival, respectively). The duration of freezing also had no effect on survival: larvae (L1's and L3's) were thawed after only 2 days and as late as 81 days postfreezing, but the viability remained similar at both intervals (57 vs. 58%, respectively).

Thawing

More larvae survived for 24 hr if the cryoprotectants were removed after thawing (21.3%) than if the larvae were left in the cryoprotectant (3.7% survival). Furthermore, a greater percentage of L3's were alive at 20 min postthawing if the worms were thawed in a saline solution rather than water and the freezing medium was replaced with either PBS or RPMI (Table I). A greater

TABLE I. *Effect of different thawing media on the survival of cryopreserved* Strongyloides stercoralis *third-stage larvae.*

Medium*	Postthawing survival (%)† 20 min	24 hr
Water	7.5 ± 9.9	1.6 ± 3.8
PBS‡	38.5 ± 13.4	7.5 ± 6.3
RPMI medium	47.3 ± 7.6	22.5 ± 8.1

* Frozen pellets of larvae were thawed in 0.5 ml of medium. Upon thawing the worms were washed in 10 ml, resuspended, and kept in the same medium.
† Percentage of motile worms ± SD.
‡ Phosphate-buffered saline.

number of larvae survived for 24 hr postthawing in RPMI than in either water or PBS (Table I).

Infectivity

Thawed L3's and L3's derived from thawed L1's were infective to puppies when injected subcutaneously (Table II). The duration of freezing had no effect on their infectivity; L3's frozen for 6 (dog 2) or 60 days (dog 3) were equally infective. The prepatent periods were within the normal range, as was the pattern of larval shedding. L1's from dog 3 grown either in charcoal–feces cultures or on agarose plates, developed to the L3 stage in quantities sufficient for infection of other dogs. Furthermore, free-living adult worms were observed on the agar plates, indicating that the thawed larvae were capable of normal heterogonic development.

DISCUSSION

Although *Strongyloides* spp. have been frozen previously and motile larvae were obtained upon thawing (Weinman and McAllister, 1947; Van Wyk et al., 1977), these larvae, when tested for infectivity in animals, proved noninfective. Because *S. stercoralis* requires a parasitic phase in

TABLE II. *Infectivity of thawed larvae for dogs.*

Dog no.	Type of larvae	No. of motile larvae injected	Prepatent period (days)	Maximum larval output (no./g feces)*
1	L3's derived from thawed L1's	1,780	14	151
2	Thawed L3's	2,230	16	2
3	Thawed L3's	6,890	17	1,500

* Dogs 1 and 2 were treated and removed from the experiment after 2 positive fecal samples, dog 3 was immunosuppressed on day 31 postinfection and the maximum larval output was seen on day 32.

its life cycle, infectivity is an important criterion for successful cryopreservation. Using infectivity as the main criterion, we have successfully preserved a strain of *S. stercoralis* using either first- or third-stage larvae as the cryopreserved form.

The use of a cryoprotectant appears to be necessary to freeze infective larvae of some species successfully (Kelly et al., 1976; James, 1985), whereas other nematodes can be frozen successfully without one (Van Wyk et al. 1977), and, occasionally, cryoprotectants can even be deleterious (Kelly and Campbell, 1974). In previous attempts to freeze *Strongyloides* spp. (Weinman and McAllister, 1947; Van Wyk et al., 1977), no cryoprotectant was used; therefore, in order to preserve the infectivity of the larvae, we decided to include a cryoprotectant in our freezing protocol. Glycerol and DMSO were chosen as cryoprotectants that provide protection after penetrating the cell, whereas dextrans (MW 9,400, 17,200,and 255,000) were chosen to represent those that function extracellularly. The choice of cryoprotectant does not appear to be critical as all those tested provided some protection, but the combination of 10% DMSO and 10% dextran (MW 9,400–17,200) was by far the best. However, because only 10% D+d was tested for its ability to preserve infectivity, we do not know if other cryoprotectants can be used for this purpose.

The presence of a cryoprotectant was not sufficient by itself to allow the L3 to survive freezing; an additional 20–60-min incubation with the cryoprotectant was also needed. The incubation may have allowed time either for the cryoprotectant to penetrate the larval cuticle or for water to be removed from the worm osmotically. The first of these explanations is based on the observation that L1's (with a different, presumably thinner, cuticle) did not require a period of incubation. The second is based on the observation that the larvae were crenated after incubation. Both of these explanations may be true and have been shown to operate in other systems (James, 1985).

Although L1's were more easily frozen than L3's (greater survival and shorter incubation times) and the surviving L1's developed into free-living adults that produced infective larvae, there are technical disadvantages to storing *S. stercoralis* as frozen L1's. There is a greater variability in the survival of L1's than L3's, especially when fast frozen, as shown by the large standard deviations in our data. One of the causes of this variability may be the large amount of particulate matter that often accompanies the L1's during collection from feces, which may change the freezing rate. These residues are reduced greatly when L3's are collected from charcoal–feces cultures and, hence, the rate of freezing is only slightly affected. Other disadvantages include the need for a controlled rate freezing apparatus to obtain the highest survival rates, and the requirement that the thawed worms be grown on agarose plates to obtain infective larvae. We have tried to inoculate charcoal–feces cultures (made with fresh feces from worm-free dogs) with thawed L1's, but our yields of L3's were very poor. For these reasons, it is less time-consuming to freeze L3's, usually 20,000 or more per vial, and inject the thawed larvae as soon as possible after removing the cryoprotectant. By freezing 20,000 L3's, sufficient infective larvae survive to provide a dose adequate for passing the infection to another dog.

The choice of a thawing medium is critical to the survival of the larvae. Worms thawed and placed directly into water have very poor survival rates although water does not appear to have such a strong adverse effect on worms that have not been frozen. We have noticed that more L3's maintained in water upon thawing have a ruptured cuticle than those maintained in RPMI. The increased survival when PBS or RPMI is used may indicate that freezing temporally impairs the worm's ability to maintain a proper osmotic balance.

Successful cryopreservation of *S. stercoralis* is best shown by the ability of the thawed larvae to produce a patent infection in susceptible puppies. With respect to this, both L1's and L3's were frozen and proved infective after thawing. Although dog #3 had a low larval output until after immunosuppression, charcoal–feces cultures made between 2 and 5 days after patency produced 3,000–5,000 L3's each day, more than sufficient to infect another dog.

Based on the results recorded above and other observations just described, the following procedure is recommended for the cryopreservation of larvae of *S. stercoralis* for routine strain maintenance:

1) Wash larvae twice in sterile distilled water.
2) Suspend 20,000 larvae in a cryo-tube containing 1 ml of the freezing mixture (1 g dextran [MW 9,400–17,200] + 1 ml DMSO + 8 ml distilled water) and incubate at room temperature for 60 min.

3) Place the cryo-tube into the vapor phase of liquid nitrogen for freezing and storage.
4) To thaw add 0.5 ml RPMI to the frozen pellet and hold at 34–37 C with occasional inversion until the pellet is melted. Transfer the thawed worms to 10 ml of RPMI, centrifuge at 150 *g* for 5–10 min, and resuspend in RPMI.
5) Inject the thawed L3's subcutaneously into a susceptible host as soon as possible after thawing.

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INFECTION OF PERUVIAN *AOTUS NANCYMAI* MONKEYS WITH DIFFERENT STRAINS OF *PLASMODIUM FALCIPARUM*, *P. VIVAX*, AND *P. MALARIAE*

William E. Collins, Jimmie C. Skinner, Marguerite Pappaioanou, J. Roger Broderson, Nancy Shi-Fong Ma*, Valery Filipski, Peggy S. Stanfill, and Laura Rogers†

Division of Parasitic Diseases and Office of Scientific Services, Center for Infectious Diseases, Centers for Disease Control, U.S. Public Health Service, Department of Health and Human Services, Atlanta, Georgia 30333

ABSTRACT: *Aotus nancymai* (karyotype I) monkeys from Peru were studied for their susceptibility to infection with *Plasmodium falciparum*, *P. vivax*, and *P. malariae*. Three strains of *P. falciparum* (Santa Lucia from El Salvador, Indochina I/CDC from Thailand, and Uganda Palo Alto) were inoculated into 38 monkeys. The results indicated that this species of *Aotus* monkey is highly susceptible to infection. The Uganda Palo Alto and the Santa Lucia strain parasites appear to be the most useful for immunologic and chemotherapeutic studies. Five strains of *P. vivax* (Chesson, ONG, Vietnam Palo Alto, Salvador I, and Honduran I/CDC) were inoculated into 28 monkeys. The Vietnam Palo Alto strain produced the highest level parasitemias ranging from 23,800 to 157,000/mm^3. Mosquito infections were obtained with the ONG, Chesson, and Salvador I strains. Two out of 6 attempts to transmit *P. vivax* via sporozoite inoculation to splenectomized monkeys were successful with prepatent periods of 39 and 57 days. Five monkeys were infected with the Uganda I/CDC strain of *P. malariae*. Maximum parasitemias ranged from 10 to 5,390/mm^3.

The search for strains of parasites and available monkeys that can be used in immunologic and chemotherapeutic studies continues. It has become apparent that pretrial testing and adaptation of the different strains is needed to determine the suitable parasite/monkey combinations that will produce reasonably high and predictable parasitemias.

Aotus nancymai monkeys from Peru are presently the most readily available of the owl or dourocouli monkeys for malaria research. Supplies of other types of monkeys have become limited due to country embargoes on their export. Most of our previous studies have used *A. lemurinus griseimembra* (karyotypes II, III, and IV) from Colombia, *A. azarae boliviensis* (karyotype VI) from Bolivia, or *A. vociferans* (karyotypes V, X, and XI) from Peru. It became apparent that each species and strain of malaria parasite must be tested in the different monkeys for (1) susceptibility to infection, (2) production of high-level parasitemias, and (3) their ability to support the production of infective gametocytes. The present report describes the results of studies initiated to determine the susceptibility of the *A. nancymai* monkeys from Peru to infection with standard strains of *P. falciparum*, *P. vivax*, and *P. malariae*.

MATERIALS AND METHODS

The *A. nancymai* monkeys were obtained from Iquitos, Peru, through the efforts of the Pan American Health Organization, Washington, D.C. These animals are members of the red-neck group, with 2n chromosome number of 54 (Hershkovitz, 1983). They have been designated as karyotype I. Adult animals are usually smaller than the Bolivian and Colombian *Aotus* monkey we have previously studied and are phenotypically and karyotypically uniform. Prior examination indicated that all animals were free of natural malarial infections. Most of the animals were splenectomized before being inoculated with the different species and strains of *Plasmodium*.

Animals were usually inoculated intravenously with heparinized parasitized blood from other types of *Aotus* monkeys. In other instances, animals were inoculated with blood that had been stored frozen with 10% glycerol over liquid N_2 and rapidly thawed. Six animals were exposed to infection with *P. vivax* either by the intravenous inoculation of sporozoites dissected from the salivary glands of infected mosquitoes into 20% fetal calf serum or by the feeding of infected mosquitoes using techniques as previously described (Collins et al., 1966).

During the course of the infection, blood films were made according to the technique of Earle and Perez (1932) and stained with Giemsa stain. When the parasitemias of *P. vivax* were high, *Anopheles freeborni*, *An. dirus*, *An. stephensi*, and/or *An. gambiae* mosquitoes were fed on the animals. Subsequent dissection and examination of the guts of these mosquitoes for

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* New England Regional Primate Research Center, Harvard University, Southborough, Massachusetts 01772.

† American Institute of Biological Sciences, Arlington, Virginia 22209.

oocysts determined if infective gametocytes had been produced.

Three strains of *P. falciparum* were examined. Infections were induced in either *A. lemurinus griseimembra* or *A. azarae boliviensis* monkeys prior to inoculation of parasitized blood into the *A. nancymai.* The Santa Lucia strain of the parasite was isolated in 1975 from a 3-yr-old Salvadoran female who presented at the Santa Lucia Clinic in La Paz Department, El Salvador (Collins et al., 1977). It has been extensively studied in Colombian and Bolivian *Aotus* monkeys (Collins et al., 1977, 1979, 1986). The strain is susceptible to the common antimalarial drugs. The Indochina I/CDC strain was isolated in 1980 from an American physician who had worked in the Khmer refugee camps along the Thailand–Kampuchean border (Centers for Disease Control, 1980). The strain is resistant to many of the antimalarial drugs including chloroquine, sulfadoxine/pyrimethamine, and quinine. It is susceptible to treatment with mefloquine. The strain has been studied in Colombian and Bolivian *Aotus* (Collins et al., 1983a, 1986) and *Saimiri* monkeys (James et al., 1985; Campbell et al., 1986). The Uganda Palo Alto strain was isolated in 1966 from a female patient presenting at the Palo Alto–Stanford Hospital with an acute infection of *P. falciparum* (Geiman and Meagher, 1967). The infection was contracted in Uganda. The strain is susceptible to chloroquine and quinine, but is resistant to pyrimethamine. It has been used extensively in chemotherapeutic studies (Schmidt, 1978a, 1978b, 1978c). We acquired the strain in 1986 from Dr. Wasim Siddiqui, University of Hawaii, as a frozen blood sample from an infected *Aotus* monkey.

Five strains of *P. vivax* were examined. The Chesson strain was isolated in 1944 from a returning serviceman who had acquired the infection in New Guinea (Ehrman et al., 1945). The strain has been extensively studied and has been passaged through numerous human volunteers, chimpanzees, and monkeys (Collins et al., 1980). The ONG/CDC strain was isolated in 1980 from a Vietnamese refugee who had spent some time on Alia and Galang islands in Indonesia. The parasite has been passaged in *Aotus* and *Saimiri* monkeys (Collins and Skinner, 1982; Collins et al., 1983b). The Salvador I strain of *P. vivax* was isolated from a human infection in the area of Cangrejera, Department of La Paz, El Salvador (Collins et al., 1972). The parasite has been passaged in human volunteers and in *Aotus* and *Saimiri* monkeys (Contacos et al., 1972; Collins et al., 1976; Campbell et al., 1983). The Honduras I/CDC strain was isolated from an American tourist who had acquired the infection in Honduras in November 1982; this strain has only received limited study (Collins et al., 1985). The Vietnam Palo Alto strain was isolated from a soldier returning from Vietnam (Geiman and Meagher, 1967). It has been extensively used in chemotherapeutic studies (Schmidt, 1978a, 1978b, 1978c). Although only slightly infective to mosquitoes, it has routinely produced very high density parasitemia in splenectomized Colombian *Aotus* monkeys.

The Uganda I/CDC strain of *P. malariae* was obtained from a Ugandan national who had come to the United States 8 yr previously from Uganda. The parasite has been passaged through a chimpanzee and numerous Bolivian *A. azarae boliviensis* monkeys (Collins et al., 1984).

RESULTS

The 3 different strains of *P. falciparum* were passaged to 38 different *A. nancymai* monkeys. The definition of a well-adapted strain of *P. falciparum* in a specific type of monkey for blood-stage vaccine trials is to obtain a maximum parasitemia of 400,000/mm^3 or greater within 14 days after intravenous inoculation of approximately 1,000,000 parasites. Preferably, this can be obtained with intact rather than splenectomized monkeys. In the present studies, 9 of the animals were splenectomized prior to inoculation and 4 monkeys were splenectomized during the course of their parasitemias. Twelve animals were inoculated with parasitized blood that had been frozen for varying periods before being thawed and inoculated intravenously into the monkeys. The prepatent periods in the animals inoculated with fresh parasitized blood ranged from 1 to 13 days, whereas the prepatent periods for those inoculated with blood that had been frozen ranged from 3 to 25 days (mean of 9.9 days). Previous infection with *P. vivax,* or with *P. vivax* and *P. malariae* in 5 splenectomized monkeys, appeared to have little effect on the maximum parasite density attained following inoculation with *P. falciparum.*

Santa Lucia strain

The maximum parasitemias in the 12 monkeys infected with this strain ranged from 8,180 for monkey AO-262 to 1,940,000/mm^3 for monkey AO-256 (Table I). The 4 monkeys splenectomized prior to inoculation had relatively low parasitemias (8,180–88,400/mm^3); these animals had recrudescences of their infections following the initial parasitemia. Four monkeys (AO-442, AO-443, AO-445, and AO-453) were splenectomized after they had experienced some parasitemia. Maximum parasitemias following splenectomy ranged from 25,500 to 872,000/mm^3. Of the 4 intact animals inoculated, 3 were infected with parasites that had been frozen. Maximum primary parasitemias ranged from 136,000 to 1,940,000/mm^3. Because 3 (AO-268, AI-256, and AI-248) of 4 monkeys had maximum parasitemias of 400,000/mm^3 or greater, this strain appeared a likely candidate for use in vaccine studies.

Indochina I/CDC strain

This strain had been successfully adapted to several different types of *Aotus* and *Saimiri* mon-

TABLE I. *Maximum parasitemias in 12* Aotus nancymai *monkeys infected with the Santa Lucia strain of* Plasmodium falciparum.

Animal no.	Passage	Splenectomy	Inoculum	Previous malaria*	Prepatent period	Maximum parasitemia: parasites/mm^3 (day)	
AO-262	1	Pre-	2.0×10^6	—	3	8,180	(26)
AO-263	1	Pre-	1.2×10^6	pv	1	88,400	(5)
						21,400	(32)
						47,800	(61)
						21,800	(89)
						5,580	(163)
AO-265	1	Pre-	2.0×10^6	—	3	19,300	(38)
						59,900	(62)
AO-389	3	Pre-	1.9×10^8	—	1	40,100	(5)
						1,860	(36)
AO-442	3	Day 11	1.4×10^6	—	13	872,000	(24)
AO-445	3	Day 11	1.4×10^6	—	13	221,000	(23)
						1,400	(59)
AO-443	4	Day 14	4.0×10^6	—	1	83,000	(15)
						216,000	(41)
						868	(67)
AO-453	4	Day 20	4.0×10^6	—	7	3,530	(13)
						25,500	(40)
AO-268	2	—	1.9×10^7†	—	19	643,000	(34)
AI-255	2	—	1.9×10^7†	—	25	136,000	(37)
						308,000	(74)
AI-256	3	—	2.1×10^8†	—	7	1,940,00	(23)
AI-248	3	—	3.1×10^4	—	11	708,000	(28)

* pv = *P. vivax*.
† Inoculated with parasitized blood that had been stored frozen.

keys and is considered a prime candidate for standardized vaccine trials, although it has a high level of resistance to several antimalarial drugs. Twenty *A. nancymai* monkeys were inoculated with the Indochina I/CDC strain (Table II). Three monkeys, splenectomized prior to inoculation (AO-401, AO-431, and AO-405), had maximum parasitemias of 1,220,000, 79,400, and 500,000/mm^3, respectively. Maximum parasitemias in the intact monkeys ranged from 3,070 (AO-390) to 560,000 (AI-300)/mm^3. In passages 2 through 7, 8 intact monkeys were inoculated with between 1 and 10×10^6 parasites. Subsequent maximum parasitemias averaged 187,000/mm^3; only 5 of the monkeys had maximum parasitemias of greater than 200,000/mm^3.

Uganda Palo Alto strain

A total of 6 monkeys were inoculated with this strain of the parasite (Table III). Two splenectomized monkeys with previous experience with *P. vivax* and *P. malariae* were inoculated with parasites that had been frozen. One of the animals (AO-432) had a maximum parasitemia of 876,000/mm^3 and was selected as the donor for subsequent passages. Linear passage through 3 intact monkeys with from 1.0×10^6 to 1.6×10^6 parasites resulted in high-level maximum parasitemias ranging from 344,000 to 620,000/mm^3. Parasites from AI-556 were frozen, stored, and subsequently thawed prior to inoculation into AI-558 (passage 5). This intact monkey had a maximum parasitemia of 1.05×10^6/mm^3 that required treatment. It was apparent that of the 3 strains examined, the Uganda Palo Alto was the one that consistently produced maximum parasitemias in the acceptable range.

Twenty-eight monkeys were inoculated with the different strains of *P. vivax* (Table IV). Attempts are being made to select strains that produce maximum parasitemias sufficiently high for producing antigens for immunologic studies and for constructing genomic libraries. In addition, several attempts were made to infect mosquitoes in the continuing search for strain/monkey combinations that can reliably support the development of infective gametocytes for comparative infectivity studies.

Chesson strain

Five splenectomized monkeys were infected with the Chesson strain—4 by the inoculation of parasitized blood, and 1 by the inoculation of sporozoites dissected from the salivary glands of

TABLE II. *Maximum parasitemias in 20* Aotus nancymai *monkeys infected with the Indochina I/CDC strain of* Plasmodium falciparum.

Animal no.	Passage	Splenectomy	Inoculum	Previous malaria*	Prepatent period	Maximum parasitemia: parasites/mm^3 (day)	
AO-401	1	Pre-	4.0×10^{8}	—	1	1,220,000	(8)
AO-431	2	Pre-	1.0×10^{7}	pv	4	280,000	(12)
AO-405	5	Pre-	1.2×10^{7}†	pv	8	500,000	(17)
AI-559	3	—	1.5×10^{8}†	—	3	79,400	(7)
AI-299	5	—	1.4×10^{8}†	—	10	136,000	(19)
AI-552	5	—	1.2×10^{8}†	—	10	53,400	(16)
AO-446	7	—	9.2×10^{7}†	—	8	3,260	(12)
AO-551	7	—	9.2×10^{7}†	—	10	24,400	(16)
AO-393	3	—	5.0×10^{4}	—	6	41,100	(16)
AO-389	1	—	5.6×10^{5}	—	9	5,770	(14)
AO-390	1	—	5.6×10^{5}	—	2	3,070	(14)
AO-438	3	—	1.0×10^{6}	—	3	75,000	(9)
AO-439	4	—	1.0×10^{6}	—	6	236,000	(13)
AI-257	6	—	1.0×10^{6}	—	2	208,000	(9)
AI-260	6	—	1.0×10^{6}	—	2	340,000	(9)
AI-555	6	—	1.0×10^{6}	—	2	128,000	(11)
AI-557	7	—	1.0×10^{6}	—	1	60,400	(9)
AO-392	2	—	3.2×10^{6}	—	3	228,000	(15)
AO-437	2	—	7.0×10^{6}	—	1	221,000	(7)
AI-300	6	—	3.4×10^{7}	—	1	560,000	(9)

* pv = *P. vivax.*
† Inoculated with parasitized blood that had been stored frozen.

infected *An. stephensi* mosquitoes. An additional 4 monkeys failed to develop detectable parasitemias following inoculation of from 9,600 to 956,000 sporozoites obtained from salivary gland dissections. Monkey AO-437 was infected by the intravenous inoculation of an estimated 1,375,000 sporozoites; the prepatent period was 57 days. The maximum parasitemias in the 5 *A. nancymai* monkeys were highest in those 2 (AO-440 and AO-441) that had not been previously infected. Both of these monkeys produced gametocytes that were infective to the different species of *Anopheles.*

ONG/CDC strain

Four monkeys were infected with the ONG/CDC strain of *P. vivax.* Three splenectomized monkeys had maximum parasitemias ranging from 6,980 to 60,400/mm^3. The lone intact monkey had a maximum parasitemia of 2,910/mm^3. Relatively high-level mosquito infections were obtained on several days by feeding on monkeys AO-453 and AO-448.

Vietnam Palo Alto strain

Eight monkeys were inoculated with the Vietnam Palo Alto strain of *P. vivax.* In other types of *Aotus* monkeys, this strain produces very high levels of parasitemia, often exceeding 200,000/mm^3. In the present study, 6 splenectomized monkeys with no previous malarial experience were inoculated for producing large quantities of parasite antigens. Maximum parasitemias ranged from 31,800 to 157,000/mm^3. Parasites were harvested by exchange transfusion and the animals were then treated to cure their infections.

TABLE III. *Maximum parasitemias in 6* Aotus nancymai *monkeys infected with the Uganda Palo Alto strain of* Plasmodium falciparum.

Animal no.	Passage	Splenectomy	Inoculum	Previous malaria*	Prepatent period	Maximum parasitemia: parasites/mm^3 (day)	
AO-432	1	Pre-	4.0×10^{8}†	pv, pm	6	876,000	(18)
AO-440	1	Pre-	4.0×10^{8}†	pv, pm	7	24,000	(16)
AI-553	2	—	1.6×10^{5}	—	1	440,000	(10)
AI-554	3	—	1.0×10^{6}	—	2	344,000	(12)
AI-556	4	—	1.0×10^{6}	—	2	620,000	(14)
AI-558	5	—	2.5×10^{6}†	—	6	1,050,000	(13)

* pv = *P. vivax,* pm = *P. malariae.*
† Inoculated with parasitized blood that had been stored frozen.

TABLE IV. *Maximum parasitemias in 28* Aotus nancymai *monkeys infected with 5 different strains of* Plasmodium vivax.

Animal no.	Splenectomy	Inoculum	Previous malaria*	Prepatent period	Maximum parasitemia: parasites/mm³ (day)		Mosquito lots +/no. dissected
Chesson strain							
AO-392	Pre-	1.1×10^7	pf	1	434	(8)	
AO-440	Pre-	5.6×10^5	—	2	14,900	(20)	2/28
AO-441	Pre-	5.6×10^5	—	4	15,800	(28)	18/38
AO-438	Pre-	1.1×10^7	pf	1	310	(12)	
AO-437	Pre-	Sporozoites	pf	57	8,740	(85)	0/27
ONG/CDC strain							
AO-262	Pre-	3.7×10^6	pf	6	6,980	(22)	
					1,670	(43)	
AO-453	Pre-	4.5×10^6	pf	4	18,200	(22)	12/14
AO-448	Pre-	2.2×10^7†	pf	12	60,400	(26)	13/15
AO-449	Pre-	1.9×10^7	—	14	2,910	(32)	
Vietnam Palo Alto strain							
AI-247	Pre-	3.5×10^7	—	1	50,000	(16)	
AI-248	Pre-	3.5×10^7	—	1	120,000	(11)	
AI-249	Pre-	3.5×10^7	—	1	31,800	(7)	
AI-250	Pre-	3.0×10^7	—	1	38,100	(5)	
AI-251	Pre-	3.0×10^7	—	1	43,300	(17)	
AI-252	Pre-	3.0×10^7	—	1	157,000	(7)	
AO-444	Pre-	3.7×10^7†	pf	5	23,800	(16)	
AO-387**	Pre-	7.0×10^6	br	1	56,400	(15)	
Salvador I strain							
AO-266	Pre-		—	3	23,800	(19)	
AO-390	Pre-	1.7×10^7†	pf	9	23,800	(25)	
AO-391	Pre-	1.7×10^7†	pf	9	27,200	(26)	
AO-431	Pre-	1.1×10^7	—	1	56,900	(8)	0/11
					37,800	(18)	
AO-432	Pre-	1.1×10^7	—	1	156,000	(15)	0/16
AO-433	Pre-	1.1×10^7	—	1	13,200	(10)	
AO-434	Pre-	1.1×10^7	—	1	64,000	(14)	0/13
AO-436	Pre-	1.1×10^7	—	1	14,100	(9)	
AI-227‡	—	1.9×10^7†	—	25	775	(38)	
AO-435	Pre-	Sporozoites	—	39	24,700	(55)	3/13
					3,660	(100)	
Honduras I/CDC strain							
AO-263	Pre-	4.5×10^6	—	1	14,700	(20)	

* pf = *P. falciparum.*

† Inoculated with parasitized blood that had been stored frozen.

‡ Second passage through *A. nancymai* monkeys; all others were primary passages.

Two monkeys previously infected with *P. falciparum* or *P. brasilianum* also produced relatively high-density parasitemias. No mosquito feedings were attempted because this strain in other types of monkeys has essentially lost its ability to produce infective gametocytes.

Salvador I strain

Ten monkeys were infected with the Salvador I strain of *P. vivax.* Maximum parasitemias in the 9 splenectomized monkeys ranged from 14,100 to 156,000/mm³. In the 5 monkeys (AO-431, AO-432, AO-433, AO-434, and AO-436) that were inoculated with high numbers of parasites to produce parasites by exchange transfusion, parasitemias ranged from 13,200 to 156,000/mm³. Although 3 of these infections were subsequently fed upon, mosquito infections were not obtained. The strain in other types of *Aotus* and *Saimiri* monkeys readily infects mosquitoes. Monkey AO-435, which was infected by the bites of infected *An. freeborni* mosquitoes, had a prepatent period of 39 days and a maximum parasitemia of 24,700/mm³. Three of 13 lots of mosquitoes that were allowed to feed on this monkey were infected.

Honduras I/CDC strain

One splenectomized monkey was infected with the Honduras I/CDC strain of *P. vivax.* The maximum parasitemia was 14,700/mm³.

A total of 5 splenectomized *A. nancymai* mon-

TABLE V. *Maximum parasitemias in 5 splenectomized* Aotus nancymai *monkeys infected with the Uganda I/CDC strain of* Plasmodium malariae.

Animal no.	Inoculum	Previous malaria*	Pre-patent period	Maximum parasitemia: parasites/mm^3	(day)
AO-440	2.8×10^5	pv	13	10	(13)
AO-441	2.8×10^5	pv	9	62	(16)
AO-262	8.0×10^6	pf, pv	1	2,880	(22)
				310	(109)
AO-437	2.3×10^6	pf, pv	5	1,670	(35)
AO-432	6.4×10^6†	pv	24	5,390	(60)

* pv = *P. vivax*; pf = *P. falciparum*.

† Inoculated with parasitized blood that had been stored frozen.

keys were infected with the Uganda I/CDC strain of *P. malariae* (Table V). All animals had previously been infected with either *P. vivax* or *P. vivax* and *P. falciparum*. The maximum parasitemias were low and ranged from 10 to 5,390/mm^3. Whether higher level parasitemias can be obtained by serial passage or by the use of previously uninfected monkeys remains to be determined.

DISCUSSION

Aotus nancymai (karyotype I) monkeys from Peru have become increasingly available for such studies. As reported here, 3 strains of *P. falciparum* will readily infect both intact and splenectomized monkeys. After passage, the Santa Lucia strain produced high-level parasitemias in 4 intact monkeys. The Indochina I/CDC strain, which has been shown to produce high-level parasitemias in *A. vociferans* (Kt V) monkeys from Peru, produced high parasitemias, but not in the hoped-for range of 400,000/mm^3, following inoculation of moderate numbers of parasites. It is possible that the inoculation of larger numbers of parasites would allow the animals to support this high level of parasitemia in the 2 wk following inoculation and thereby fulfill the established criteria. The Uganda Palo Alto strain of *P. falciparum* appears from these studies to have the greatest potential of the strains thus far examined to produce such high-level parasitemias in intact monkeys, even following inoculation of parasites that have been frozen.

In certain studies, it may be possible to immunize monkeys while they are intact and subsequently splenectomize them before challenge. If monkeys thus treated were capable of a satisfactory immunologic response to infection, any of the 3 strains might be useful.

Based on the results of these studies, it is proposed that the Uganda Palo Alto strain of *P. falciparum* produces parasitemias in *A. nancymai* monkeys sufficiently high to be considered for blood-stage vaccine trails in intact monkeys and that the parasitemias are high enough for other immunologic and chemotherapeutic studies. Further studies may be needed to establish the usefulness of the Santa Lucia strain.

The *A. nancymai* monkeys are readily susceptible to all the different strains of *P. vivax* tested. Maximum parasitemias are within the range of those seen with infections with other types of *Aotus* and *Saimiri* monkeys. Although the number of mosquito feeding studies were minimal, it was shown that at least 3 of the strains produced infective gametocytes in these animals. This species of *Aotus* appeared well suited for immunologic, vaccine, and chemotherapeutic studies with *P. vivax*.

The preliminary studies with *P. malariae* in these animals indicated that monkeys previously infected with *P. vivax* or *P. vivax* and *P. falciparum* were susceptible to infection. Because linear passage of malaria parasites in different monkeys has usually produced increasing adaptation and higher parasitemias, linear passage in *A. nancymai* monkeys, particularly in those with no previous malarial experience, should result in higher level parasitemias that could prove useful in the production of antigens. The application of this model for vaccine or chemotherapeutic studies remains to be determined.

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Scott Lyell Gardner*, Robert L. Rausch†, and Otto Carlos Jordan Camacho‡
Department of Biology, The University of New Mexico, Albuquerque, New Mexico 87131

ABSTRACT: Among approximately 2,000 mammals examined for helminths in various regions of Bolivia during 1983–1987, cysts of *Echinococcus vogeli* Rausch and Bernstein, 1972, were found in a single paca, *Cuniculus paca* L., collected at La Laguna, Departamento de Santa Cruz (lat. 16°36′W; long. 62°42′S). This record, the first from Bolivia, represents a considerable extension of the known geographic range of this species in South America. Upon analysis of the morphologic characteristics of the protoscoleces derived from the cysts, the sizes of rostellar hooks from the material from the paca were found to be well within the ranges reported in previous studies. Statistical analysis of frequency distributions of hook characteristics revealed some deviations from normality. These results indicate that parametric statistics should be applied with caution in analyses of inter- and intraspecific variation of morphologic characteristics of hooks of metacestodes of the genus *Echinococcus*.

Investigations during the last decade have shown that *Echinococcus vogeli* Rausch and Bernstein, 1972, has an extensive geographic range in Central America and South America. Records based mainly on the identification of the larval stage (metacestode) from wild rodents and from cases of polycystic hydatid disease in man have documented its occurrence in Panama, Colombia, Ecuador, Venezuela (Rausch and Bernstein, 1972; D'Alessandro et al., 1979; Rausch et al., 1981), and in Brazil (from Serra do Navio and Belem in the north and, based on cases of polycystic hydatid disease, from the State of Acre in the Amazonian region, and from Monte Azul Paulista in the State of Sao Paulo) (Rausch et al., 1984; Meneghelli, 1985; Meneghelli et al., 1986). The distribution of this cestode no doubt coincides with that of its natural hosts, the bush dog, *Speothos venaticus* (Lund), and rodents of the family Dasyproctidae. The paca, *Cuniculus paca* L., appears to be the most important intermediate host. Thus, *E. vogeli* may be expected to occur from about the level of the Isthmus of Tehuantepec, in southern Mexico, southward to Bolivia, Paraguay, and southern Brazil.

In the course of fieldwork in Bolivia during the years 1984–1987, we (S.L.G. and O.C.J.C.) examined more than 2,000 mammals for helminths. These specimens, consisting mainly of rodents, were collected at several localities within all of the major habitat types represented in that country. Of rodents previously reported to serve as intermediate hosts of *E. vogeli*, 4 pacas, 7 agoutis (*Dasyprocta* spp.), and 158 spiny rats (*Proechimys* spp.) were examined. The larval stage of *Echinococcus* sp. was found in 2 pacas.

The present paper reports the first record of *Echinococcus vogeli* in Bolivia.

MATERIALS AND METHODS

All mammals were necropsied promptly after death, to avoid possible effects of autolytic changes on the helminths. The larval stage of *Echinococcus* sp. was found in the liver of 2 pacas, collected at La Laguna, 10 km N of San Ramon, Departamento de Santa Cruz, Bolivia (lat. 16°36′S; long. 62°42′W). Cysts of *Echinococcus* were removed intact from the hepatic tissue, injected with a small quantity of undiluted formalin, and preserved in a 10% formalin solution. In the laboratory, the cysts were transected, and brood capsules were stained in Semichon's acetic carmine. Protoscolices were separated and mounted in Canada balsam with sufficient pressure applied to the cover-glass to cause the rostellar hooks to lie flat. Rostellar hooks were measured by the method of Rausch et al. (1978), with the use of an oil immersion objective (1,000×). To assess normality and to determine the suitability of the data for parametric statistical tests, normal probability plots were constructed and examined for linearity; extent of skewness and kurtosis also were determined (Table I). Correlations were determined from a Pearson product moment correlation matrix (Table II). Levels of statistical significance were set at $P \leq 0.05$ prior to collection of data. Information concerning normality and correlations among morphological char-

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* To whom reprint requests should be addressed.
† Division of Animal Medicine, SB-42 University of Washington, Seattle, Washington 98195.
‡ Zoo Santa Cruz, Fauna Sudamericana, Casilla 754, Santa Cruz, Bolivia.

TABLE I. *Summary of mensural data of hooks from protoscolices recovered from metacestodes of* Echinococcus vogeli *from the liver of a paca,* Cuniculus paca, *in the Departamento de Santa Cruz, Bolivia.*

Character	n	Min	Max	Mean	Range	S	S²	Skewness g_1	Kurtosis g_2
Large hooks									
Total length	100	37	44	39.83	7	1.49	1.22	0.69	0.64
Handle length	100	13	18	15.50	5	0.79	0.89	0.47	1.19*
Guard width	100	10	16	12.90	6	1.60	1.26	0.006	−0.54
Blade length	100	20	33	24.29	13	2.23	1.49	1.64*	10.53*
Small hooks									
Total length	100	2.7	35	32.59	4	1.25	1.12	0.37	−0.58
Handle length	100	13	19	16.34	6	1.36	1.16	0.08	0.64
Guard length	100	8	13	10.33	5	1.03	1.02	−0.28	0.22
Blade length	100	13	23	16.39	10	1.97	1.40	1.04*	3.99*

* Indicates statistically significant deviation from a normal distribution (i.e., H_0:g_1 = 0) ($P \leq 0.05$).

acters of the hooks from the protoscolices has been included to facilitate future comparisons. All measurements are given below in μm, unless otherwise stated.

RESULTS

In the first paca 3 cysts were present in the liver. They were subspherical in shape and about 15–20 mm in greater diameter. The comparatively large brood capsules were rather few and scattered on the surface of the germinal layer. The cysts from the second paca were sterile, and the cestode therefore was not identifiable.

Based on form, proportions, and dimensions of the rostellar hooks, we identified the larval cestode as *E. vogeli* (see comparative measurements, Table III). Some brood capsules contained hooks that were not yet fully developed.

Lengths of hooks reported previously (Rausch et al., 1978) were as follows: large hooks (n = 313), 39.1–45.6 ($\bar{x}$ = 41); small hooks (n = 283), 30.4–36.9 ($\bar{x}$ = 33). For the present material, lengths of large hooks judged to be fully developed (n = 100) ranged from 37 to 44 ($\bar{x}$ = 40); those of small hooks (n = 100), 2.7 to 35 ($\bar{x}$ = 32.6) (Figs. 1, 2). These dimensions were well within the range previously reported for *E. vogeli* by Rausch et al. (1978).

In proportions and dimensions, the hooks from the Bolivian material differed from those of the larval *E. oligarthrus* (Diesing, 1863). Large hooks of *E. oligarthrus* ranged from 29.1 to 37.9 ($\bar{x}$ = 32); small hooks, 22.6 to 29.5 ($\bar{x}$ = 25.9) (Rausch et al., 1978).

Dimensions of hooks from the Bolivian material exhibited relatively few correlations (Table III). Statistically significant correlations were evident between lengths of large hooks and width of guard of large hooks, and lengths of large hooks and length of blade of small hooks. A significant negative correlation was evident between length of handle and width of guard of large hooks. Length of blade of small hooks was found to be positively correlated with both guard width of large hooks and length of small hooks, and negatively correlated with length of handle.

The observed frequency distributions obtained from measurements of material from Bolivia are summarized in Table I. Only those values significantly different from a normal distribution ($P \leq 0.05$) are discussed. The frequency distribution of measurements of length of handle of large hooks is slightly leptokurtic, whereas the distribution of length of blade of large hooks is significantly skewed toward the larger values and is leptokurtic (Table I). The

TABLE II. *Summary of lengths of hooks from protoscolices of 2 species of* Echinococcus *from natural infections of* Cuniculus paca *in South America.*

Species	Large hooks n	Large hooks Range	Large hooks Mean	Small hooks n	Small hooks Range	Small hooks Mean	Source of material
E. vogeli	100	[illegible]	39.83	100	27–35	32.59	Liver of paca, Dept. Santa Cruz, Bolivia
E. vogeli	313	39.1–45.6	41.08	283	30.4–36.9	32.98	Liver of paca (Rausch et al., 1978)
E. oligarthrus	–	30.4–33.9	32.00	50	24.3–28.7	25.90	Muscle of paca (Rausch et al., 1978)

TABLE III. *Pearson product moment correlations among characters of hooks from protoscolices of* Echinococcus vogeli *from* Cuniculus paca *in the department of Santa Cruz, Bolivia. For all measurements n = 100.*

Characters	Large hook length	Large hook handle length	Large hook guard width	Large hook blade length	Small hook length	Small hook handle length	Small hook guard width	Small hook blade length
Large hook length	1.0	—	—	—	—	—	—	—
Large hook handle length	0.199*	1.0	—	—	—	—	—	—
Large hook guard width	0.087	0.081	1.0	—	—	—	—	—
Large hook blade length	0.697*	−0.443*	0.032	1.0	—	—	—	—
Small hook total length	0.327*	0.086	0.091	0.155	1.0	—	—	—
Small hook handle length	0.169	0.049	−0.107	0.070	0.397*	1.0	—	—
Small hook guard width	0.046	0.050	0.192	0.003	0.168	0.186	1.0	—
Small hook blade length	0.074	−0.004	0.279*	0.018	0.410*	−0.476	−0.056	1.0

* Indicates significant correlation between characters at $P \leq 0.05$.

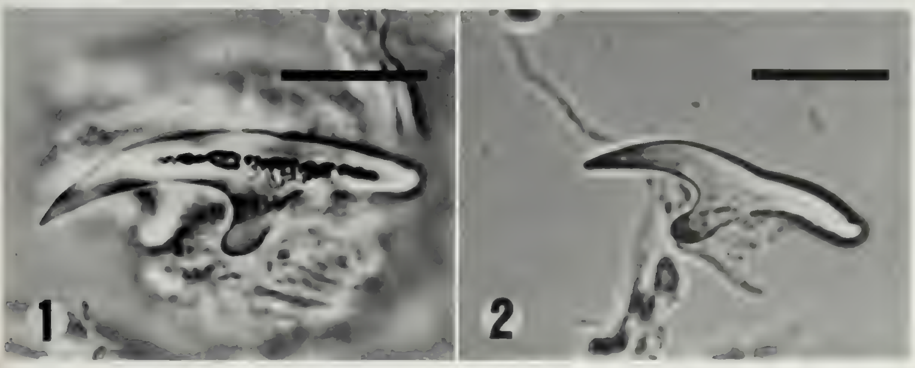
frequency distribution of length of blade of small hooks is also positively skewed and leptokurtic (Table I).

DISCUSSION

The deviations from normality evident in the frequency distributions of those characters mentioned above require that some transformations may be necessary before parametric statistical tests can be applied for comparative purposes. Some hooks that were measured may not have been fully developed, possibly causing the observed deviations. Multivariate statistical analyses (less susceptible to deviations from normality than univariate tests) may allow more complete comparisons of geographic and host-induced variation within and among species of *Echinococcus* in the neotropics.

The northeastern lowlands of the Departamento de Santa Cruz consist of palm-nut savanna interspersed with rather dense gallery forest (Hershkovitz, 1969; Unzueta, 1975). At the locality where the pacas were collected, the vegetation was a mixture of primary and secondary growth, semitropical deciduous forest. Some areas were heavily grazed by cattle. The northeastern part of the Departamento de Santa Cruz is recognized as a zone of faunistic and floristic transition where the southern elements of the Chaco thorn forest intergrade with the more humid tropical and subtropical deciduous forests of the Amazon basin (Unzueta, 1975; Mercado, 1985).

Most people in the region subsist in part by hunting with use of dogs, and some domestic cats are kept (Dickerman, pers. comm.). The paca is a preferred food resource, and the viscera of the rodents are usually discarded where they are readily available to dogs. Living conditions and close association with the dogs would seem to favor infection of man by *E. vogeli,* as in Colombia and other countries (D'Alessandro et al., 1979). The lack of any records of human cases of polycystic hydatid disease in Bolivia might be



FIGURES 1, 2. **1.** Large hook from protoscolex of *E. vogeli.* Scale bar = 16 μm. **2.** Small hook from protoscolex of *E. vogeli.* Scale bar = 16 μm.

attributable to the limited availability of diagnostic services.

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CHARACTERIZATION OF CROSS-REACTIVE BLOOD-STAGE ANTIGENS OF THE *PLASMODIUM CYNOMOLGI* COMPLEX USING ANTI-*PLASMODIUM VIVAX* MONOCLONAL ANTIBODIES

Kamal K. Kamboj, John W. Barnwell, Ruth S. Nussenzweig, and Alan H. Cochrane*
Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10010

ABSTRACT: Five out of 18 monoclonal antibodies (moAB's) produced against blood stages of a Brazilian (Belem) strain of *Plasmodium vivax* were shown to cross-react with all of the 11 strains of the *P. cynomolgi* complex that were assayed. The 5 moAB's produced 3 different patterns of immunofluorescence, identical for both *P. vivax* and *P. cynomolgi*. Three of these moAB's appeared to react with antigens associated with the cytoplasm or membranes of infected erythrocytes. By Western blot analysis, 2 of these 3 moAB's identified an antigen with an apparent molecular weight of 31 kDa in extracts of parasitized erythrocytes of both species; the third of these moAB's reacted with an antigen with an apparent molecular weight of 95 kDa. By immunofluorescence, the 2 other moAB's reacted only with parasites at all developmental stages. The target antigen of these 2 moAB's was not identified. Immunoradiometric assays indicated that the moAB's are directed against 3 or possibly 4 distinct nonrepetitive epitopes. None of the moAB's inhibited merozoite invasion or growth of the parasites in an *in vitro* culture system of the Berok strain of *P. cynomolgi*.

A single immunodominant species-specific antigen is present on the surface membrane of malaria sporozoites (Nussenzweig and Nussenzweig, 1985). Studies on sexual and asexual blood-stage antigens have focused primarily on *Plasmodium falciparum* because it can be continuously cultured *in vitro* (Trager and Jensen, 1976) and is the most lethal of the human malarial infections. An ever-increasing number of antigens is now being identified and characterized for asexual blood stages of *P. falciparum*, indicating the highly complex antigenic composition of asexual parasites (Miller et al., 1986).

Monoclonal antibodies (moAB's) produced against asexual parasites of one malaria species have, in certain instances, reacted with other malaria species suggesting that certain antigens or at least some of their epitopes might be common to different species of *Plasmodia*. For instance, some moAB's produced against asexual blood stages of *P. vivax* reacted by immunofluorescence with blood stages of *P. ovale, P. falciparum,* and *P. cynomolgi,* and several moAB's produced against *P. ovale* were reactive with *P. vivax, P. falciparum,* and *P. malariae* (Andrysiak et al., 1986). Observations of cross-reactivity do not necessarily indicate a sharing of epitopes, and certainly do not in all instances imply common biological properties and functions (Zavala et al., 1986).

Recently, a series of moAB's was produced by one of us against asexual blood parasites of the Belem strain of *P. vivax* (Barnwell, 1986). We screened these moAB's for reactivity with members of the *P. cynomolgi* complex because a series of observations indicated a close evolutionary relationship between *P. vivax* and *P. cynomolgi* (Coatney et al., 1971). By immunofluorescence 5 of the moAB's were strongly cross-reactive with blood stages of the 11 strains of the *P. cynomolgi* complex that we tested. Electrophoretic mobilities of the antigens of both *P. vivax* and various strains of the *P. cynomolgi* complex, recognized by the anti-*P. vivax* moAB's, were determined by Western blot analysis. These moAB's were also used in other immunoassays to further characterize their target antigens and to attempt to define a possible functional role for these antigens.

MATERIALS AND METHODS

Parasites

Rhesus monkeys (*Macacca mulatta*) were infected with 11 different strains of the *P. cynomolgi* complex, namely, NIH, Mulligan, PT, London, Langur, Gombak, Ceylon, Smithsonian, RO, Berok, and Cambodian (Coatney et al., 1971; Cochrane et al., 1986).

Cultivation and synchronization of parasites

The Berok strain of *P. cynomolgi* was cultured as described previously (Nguyen-Dinh et al., 1981). For merozoite invasion and growth assays, parasites were concentrated to schizont and ring stages, respectively,

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* Author to whom correspondence should be addressed.

by density gradient centrifugation in Percoll (Barnwell et al., 1982).

Immunization of mice and production of hybridomas

BALB/c mice were immunized by multiple inoculations of approximately 10^8 mixed blood-stage parasites of the Belem strain of *P. vivax* (Barnwell et al., 1988). Hybrid cells were screened for antibody production by indirect immunofluorescence using air-dried blood stages of *P. vivax*. MoAB's were purified from mouse ascitic fluid using an Affi-Gel protein A MAPS column (Bio-Rad, Richmond, California).

Indirect immunofluorescent antibody test

Parasitized erythrocytes in fetal calf serum were placed in wells of Teflon-coated slides, dried at room temperature, and held over desiccant under vacuum. Prior to use, the parasites were fixed with chilled acetone, washed with phosphate-buffered saline (PBS), and incubated with culture supernatant or purified moAB for 30 min at 37 C. They were further incubated with fluoresceinated anti-mouse IgG (Meloy) at 37 C for 30 min, washed, and mounted in 90% glycerol, 10% PBS, containing p-phenylenediamine (Sigma) to reduce bleaching (Johnson and de C. Nogueira Araujo, 1981).

Wet preparations of parasitized red blood cells were incubated with purified moAB in PBS containing 1% bovine serum albumin (BSA), washed, and incubated with fluoresceinated anti-mouse IgG. The preparations were mounted in PBS (Marsh et al., 1986) or in PBS containing 0.5% formalin (Barnwell et al., 1983).

Metabolic labeling of parasites, immunoprecipitation, and SDS-gel electrophoresis

Plasmodium cynomologi (Berok) cultures were biosynthetically labeled with ^{35}S-methionine and immunoprecipitated with moAB's following the procedure of Schofield et al. (1986). After electrophoresis (Laemmli, 1970), the gels were fixed, impregnated with Enhance, and fluorographed at −70 C.

Western immunoblot

Parasite extracts were prepared by placing ca. 1×10^6 freshly isolated infected erythrocytes, containing parasites of various stages, in 100 μl of sample buffer. After boiling for 3 min and centrifugation at 10,000 *g* for 10 min, 30 μl of extracts were slab electrophoresed using a 10% running gel (Laemmli, 1970). Western blot analysis (Towbin et al., 1979) was performed as previously described (Cochrane et al., 1984).

Reciprocal inhibition of binding of moAB's

Wells of microtiter plates were coated with poly-L-lysine (1 mg/ml) in PBS for 1 hr at room temperature. Parasite antigens were extracted in PBS containing 0.5% Nonidet P40, 1% BSA, and protease inhibitors as detailed above. Thirty μl of extract (ca. 25,000 parasites) were placed in wells of the microtiter plates at room temperature for 1 hr. Thereafter, this assay was performed as described (Zavala et al., 1983). MoAB's used in this and the subsequent assay were I^{125}-labeled by the Bolton Hunter protocol (Bolton and Hunter, 1973).

Two-site immunoradiometric assay (IRMA)

This assay was essentially performed as described (Zavala et al., 1983). Parasite antigens were extracted as described above, diluted 1:5 in PBS-BSA, and centrifuged at 10,000 *g* for 10 min. Extract equivalent to about 25,000 parasites was placed in each well.

Merozoite invasion and *in vitro* growth assays

Purified moAB's were dialyzed against 4 changes of 1,000× volume of RPMI-1640 containing 30 mM HEPES, and passed through a 0.22-μm surfactant-free filter. Each moAB was added in concentrations ranging from 50 to 500 μg/ml to triplicate wells of microtiter plates containing schizont-synchronized cultures of *P. cynomolgi* (Berok). Each well contained moAB in 100 μl RPMI-1640, 10% human O+ serum, 30 mM HEPES, and 1 μg/ml gentamycin sulfate. Hematocrits and starting parasitemias were 2.5% and 1%, respectively. Control wells were identical in all aspects but contained noncross-reacting moAB. The cultures were incubated for 18 hr at 37 C in 90% N_2, 5% CO_2, and 5% O_2 until the majority of the schizonts had ruptured. Parasitemias were determined from Giemsa-stained thin films.

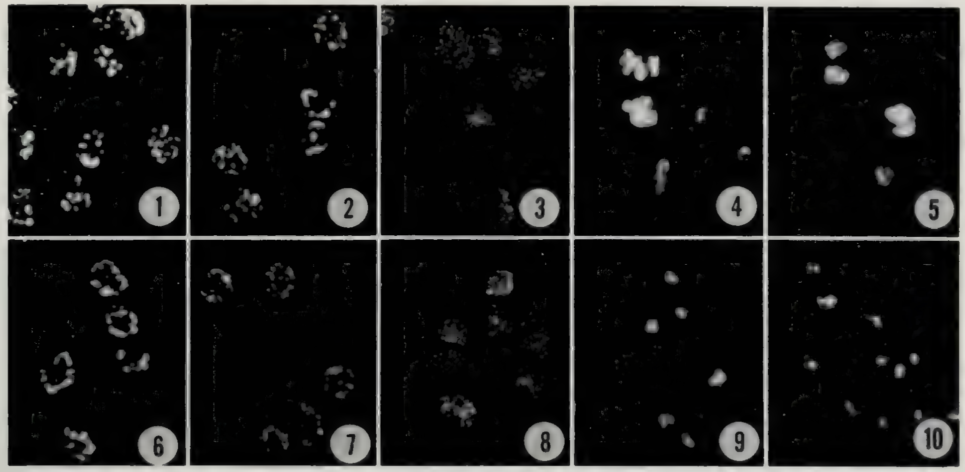
To determine whether the moAB's were inhibiting the *in vitro* maturation of the parasites, moAB's were added at a final concentration of 500 μg/ml to cultures with a ring-stage parasitemia of approximately 1%. After 32 hr of culture, thin films were made and Giemsa stained, and parasitemia and stage of development of the parasites determined.

RESULTS

Patterns of immunofluorescence produced by moAB's

Five of 18 moAB's produced against asexual blood parasites of *P. vivax* (Belem strain) reacted by immunofluorescence with dried and acetone-fixed blood stages of 11 strains of the *P. cynomolgi* complex. Titers of 3 of the moAB's, 4C12B10, 1E9D5, and 1C2F10, were essentially the same with all strains of *P. cynomolgi* and with *P. vivax*. Reactivity was observed with 10 ng/ml of purified immunoglobulin. Titers of the other 2 moAB's, 1H4B6 and 1A3B4, were considerably higher for *P. vivax* than for the *P. cynomolgi* strains. These moAB's were still reactive at concentrations of 10 ng/ml with *P. vivax* and 10 μg/ml with *P. cynomolgi*.

The 5 moAB's (all IgG_1) produced the same 3 distinct patterns of immunofluorescence with both *P. cynomolgi* and *P. vivax* (Figs. 1–10). Three of these moAB's, 4C12B10, 1A3B4, and 1H4B6, appeared to react with a cytoplasmic component or the membrane of infected erythrocytes, at all stages of growth of the parasites; moAB's 4C12B10 and 1A3B4 showed a coarse, irregular, and diffuse distribution of parasite antigen, while moAB 1H4B6 produced a uniform speckled pattern. The other 2 moAB's, 1C2F10 and 1E9D5,



FIGURES 1–10. Immunofluorescent patterns of anti-*P. vivax* moAB's (1, 6, moAB 4C12B10; 2, 7, moAB 1A3B4; 3, 8, moAB 1H4B6; 4, 9, moAB 1C2F10; 5, 10, moAB 1E9D5) with air-dried asexual blood parasites of *P. vivax* (1–5) and the Berok strain of *P. cynomolgi* (6–10). The *P. vivax* parasites are at various stages of development; the *P. cynomolgi* parasites are ring stages only. For both *P. vivax* and *P. cynomolgi*, the staining patterns of the 5 moAB's appear identical. MoAB's 4C12B10, 1A3B4, and 1H4B6 react with antigens associated with the membranes or cytoplasm of infected erythrocytes; moAB's 1C2F10 and 1E9D5 react only with the parasites at all stages of development.

reacted only with the parasites, at all stages of development.

None of the 5 moAB's reacted with the membranes of wet preparations of *P. cynomolgi* (Berok)-parasitized erythrocytes by indirect immunofluorescence with or without postfixation of the cells.

Identification of the *P. cynomolgi* antigens

By Western blot (Fig. 11), moAB's 4C12B10 and 1A3B4 reacted with a protein, of identical electrophoretic mobility, in extracts of both *P. cynomolgi* and *P. vivax* (apparent molecular weight 31 kDa). MoAB 1H4B6 recognized a protein with a molecular weight of approximately 95 kDa present in both *P. cynomolgi* and *P. vivax* extracts. MoAB's 1C2F10 and 1E9D5 failed to recognize an antigen by immunoblot or by immunoprecipitation following metabolic labeling of the parasites with ^{35}S-methionine.

MoAB's recognize distinct nonrepetitive epitopes

The results of reciprocal inhibition of binding indicate that the 5 *P. vivax* moAB's recognize at least 3 and possibly 4 distinct epitopes in the *P. cynomolgi* complex (Table I). The fact that moAB's 4C12B10 and 1A3B4 only partially inhibited the binding of each other in the assay suggests that they may be recognizing 2 different epitopes each associated with the 31-kDa protein.

We also used a 2-site IRMA to determine if the epitopes recognized by the moAB's were repetitive. Using the same moAB in both the solid and fluid phases indicated that the epitopes recognized by the moAB's are nonrepetitive in the *P. cynomolgi* strains (results not shown).

MoAB's fail to inhibit parasite reinvasion and maturation

Each of the 5 moAB's was added at a concentration of 50–500 μg/ml to *P. cynomolgi* (Berok) schizont-synchronized cultures. After 18 hr of incubation, there were no detectable differences in the levels of parasitemia or the stages of development when compared to control cultures (Table II).

When added at a final concentration of 500 μg/ml to ring-stage-synchronized cultures of *P. cynomolgi* (Berok), the moAB's produced no inhibitory effect on parasite growth and maturation after 32 hr of incubation (results not shown).

DISCUSSION

Results of the present study indicate similarities between certain blood-stage antigens of *P. cynomolgi* and *P. vivax* and support the use of the *P. cynomolgi*–rhesus monkey system as an

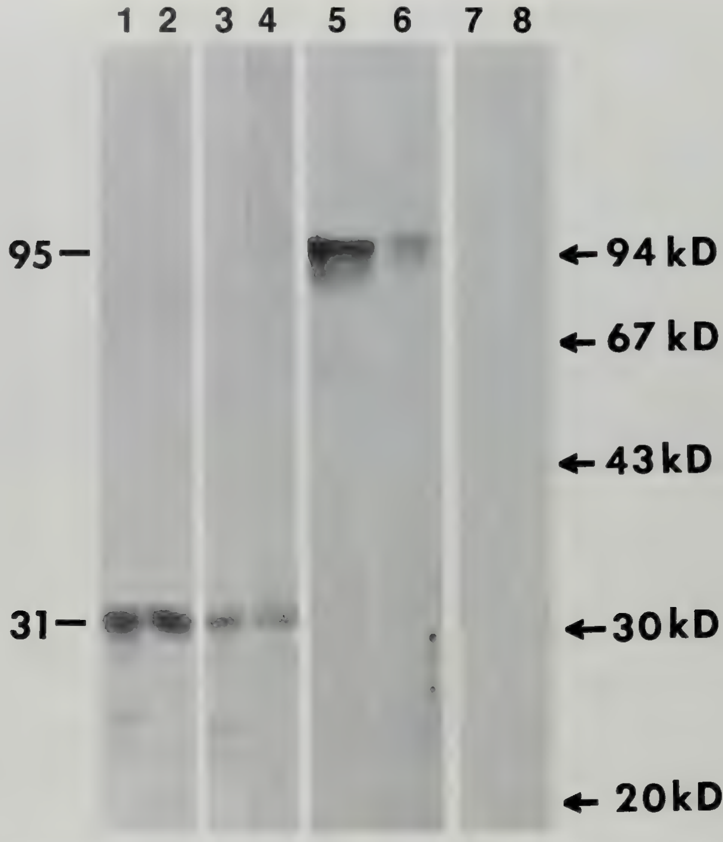


FIGURE 11. Western blot of extracts of asexual blood stages of *P. vivax* (Belem) and *P. cynomolgi* (Berok) using moAB's produced against *P. vivax*. MoAB's 4C12B10 and 1A3B4 detected a protein with an apparent molecular weight of 31 kDa common to both *P. vivax* (lanes 1 and 3, respectively) and *P. cynomolgi* (lanes 2 and 4, respectively). MoAB 1H4B6 identified an antigen with an approximate molecular weight of 95 kDa in both *P. vivax* (lane 5) and *P. cynomolgi* (lane 6) extracts. These proteins were not recognized in *P. vivax* and *P. cynomolgi* extracts by moAB 3D11 (lanes 7 and 8, respectively) produced against *P. berghei* sporozoites.

experimental model that facilitates characterization of some blood-stage antigens of *P. vivax*. The Berok strain of *P. cynomolgi* is one of the few primate malaria parasites that can be continuously cultured *in vitro* (Nguyen-Dinh et al., 1981). The ease of cultivation and synchronization makes it a good system for assaying moAB's for identification of protective antigens. The readily obtainable large numbers of parasites also facilitate DNA studies and the cloning of genes encoding relevant parasite proteins.

Whether the protective blood-stage antigens of members of the *P. cynomolgi* complex are strain specific, as has been found with the protective circumsporozoite proteins (Cochrane et al., 1986), remains to be determined. Early observations of Voller and Rossan (1969) suggest that protective immunity does not exist between blood stages of *P. cynomolgi bastianellii* and *P. cynomolgi ceylonensis,* although they observed protective immunity between *P. cynomolgi bastianellii* and *P. cynomolgi cynomolgi.* The failure of our moAB's to inhibit the *in vitro* growth of the parasites does not exclude their having an inhibitory effect *in vivo.* Furthermore, other epitopes that induce protective immunity may be present on the same or other antigens.

The distribution of antigen recognized by moAB 1H4B6 resembles that of Schuffner's dots. Immunochemical and ultrastructural studies using several moAB's against the 95-kDa antigen, including 1H4B6, indicate that they react with

TABLE I. *Reciprocal inhibition of binding of anti-*Plasmodium vivax *moAB's to* P. cynomolgi *(Berok) blood-stage antigens.*

Cold inhibitor moAB	Percent inhibition of binding of radiolabeled moAB to *P. cynomolgi* (Berok) antigen				
	4C12B10	1A3B4	1H4B6	1E9D5	1C2F10
4C12B10	100	54	19	11	0
1A3B4	22	100	22	20	18
1H4B6	3	7	100	21	13
1E9D5	7	5	11	100	106
1C2F10	14	11	5	97	100

TABLE II. *Merozoite invasion assay with* P. cynomolgi *(Berok) cultured in the presence of anti-*P. vivax *moAb's.**

moAB (500 μg/ml)	Infected RBC (%)	Stage (%)		
		Ring	Late trophozoites	Schizonts
No moAB, 0 hr	0.6	11	72	17
No moAB, 18 hr	1.4	76	12	12
4C12B10	1.5	74	16	10
1A3B4	1.6	73	14	13
1H4B6	1.2	68	20	12
1E9D5	1.8	76	13	11
1C2F10	1.4	77	11	12
3D11†	1.4	74	10	16

* All cultures were terminated 18 hr after addition of moAB.
† Anti-*P. berghei* sporozoite moAB.

caveola vesicles in the membranes of *P. vivax*-parasitized erythrocytes (Barnwell and Aikawa, pers. comm.). Aikawa et al. (1975) have suggested that there is a morphological relationship between Schuffner's dots and caveola vesicles. In contrast, the immunofluorescent staining pattern of moAB's 1A3B4 and 4C12B10 suggests that they might be directed against the cleft structures present in the cytoplasm of red blood cells infected with *P. vivax* or *P. cynomolgi* (Aikawa et al., 1975).

The reason for the failure of 2 of the moAB's, 1C2F10 and 1E9D5, to detect antigen by Western blot, under reducing and nonreducing conditions, and also by immunoprecipitation is not readily apparent. Perhaps the epitope recognized by these moAB's is conformational and may have been denatured by the sample buffer used for preparing the parasite extract. Alternatively, these moAB's may have low binding affinities.

We recently generated a series of moAB's against asexual stages of *P. cynomolgi* (Berok) of which 50% reacts with *P. vivax*. Several of these moAB's produce a speckled pattern of immunofluorescence and identify a 95-kDa protein in extracts of both *P. vivax* and *P. cynomolgi*. Some of the anti-*P. cynomolgi* moAB's produce a coarse and irregular speckled pattern of immunofluorescence and recognize a protein in extracts of *P. vivax* and *P. cynomolgi* that has the same electrophoretic mobility as the 31-kDa protein recognized by the anti-*P. vivax* moAB's 4C12B10 and 1A3B4 (Kamboj et al., unpubl. data).

A close evolutionary relationship between *P. vivax* and *P. cynomolgi* has previously been recognized, based on parasite morphology and course of infection in the host (Coatney et al., 1971). Recent nucleotide sequence data of the entire CS genes of 6 strains of the *P. cynomolgi* complex (Enea et al., 1986; Galinski et al., 1987) and of *P. vivax* (Arnot et al., 1985) indicate a very high degree of homology among these parasites, except in the immunodominant repeat region. Within the repeat region, there is a high degree of homology between *P. vivax* and the Berok strain of *P. cynomolgi* (Galinski et al., 1987). By immunofluorescence, the 5 anti-*P. vivax* moAB's also cross-reacted with *P. simium*, a simian malaria parasite, whereas 4 of the 5 moAB's reacted with another simian parasite, *P. knowlesi*; none of the 5 *P. vivax* moAB's reacted with blood stages of *P. falciparum* or with *P. brasilianum* (Barnwell, 1986).

The results of our present study, indicating that certain blood-stage proteins of *P. vivax* and *P. cynomolgi* appear to be analogous, strengthen the acknowledged evolutionary relationship between these 2 malaria species.

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INFLUENCE OF IMMUNIZING DOSE AND PRESENCE OR ABSENCE OF ADULT WORMS ON THE DEVELOPMENT OF RESISTANCE TO *NEMATOSPIROIDES DUBIUS* CHALLENGE INFECTIONS OF MICE

F. Javier Enriquez*, Raymond H. Cypess, and Donald L. Wassom†
Department of Microbiology, Immunology, and Parasitology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

ABSTRACT: H-2 congenic strains expressing resistant (H-2^q, H-2^f) or susceptible (H-2^k) haplotypes were compared for their ability to resist challenge infection with *N. dubius* following a 6- or 14-day ivermectin-abbreviated immunizing infection. B10.BR mice (H-2^k) were considerably more resistant to infection when the priming interval was shortened from 14 to 6 days. B10.Q (H-2^q) and B10.M (H-2^f) mice resisted challenge regardless of which immunization regimen was used. The influence of parasite numbers on the response to challenge was studied by comparing infections in resistant DBA/1 (H-2^q) and susceptible CBA/J (H-2^k) mice that differ at both H-2 and non-H-2 genes. DBA/1 mice, immunized with 50 or 150 L3 of *N. dubius* for 14 days, resisted challenge, whereas mice receiving 300 worms did not. In contrast, CBA/J mice failed to resist challenge at all priming doses tested. When the immunizing infection was shortened from 14 to 6 days, DBA/1 mice resisted challenge regardless of priming dose and CBA/J mice resisted challenge only when the highest dose of 300 worms was used for priming. The data suggest that susceptible strains of mice may be preferentially immunosuppressed, particularly at low infective doses, and that suppression is associated with adult worms present in the lumen of the small intestine.

Nematospiroides dubius, an intestinal nematode parasite of the mouse, is an excellent laboratory model for studying the factors involved in development of resistance to reinfection. Primary infections are retained for up to 10 mo after which expulsion occurs, probably due to senescence of the adult worms (Pritchard et al., 1983); subsequent infections, however, may be resisted by the host and this ability appears to be genetically controlled (Behnke and Robinson, 1985; Enriquez et al., 1987). Inbred strains of mice differ markedly in their ability to resist a challenge infection after 1 immunizing infection. For example, LAF1 mice exhibit strong resistance after a single immunizing infection, whereas CBA mice fail to develop resistance even after 2 immunizing infections (Jacobson et al., 1982). A number of different protocols have been used effectively by researchers to stimulate acquired immunity to *N. dubius* challenge infections. These include: (A) Extraintestinal (intravenous [iv], intraperitoneal [ip], or subcutaneous [sc]) inoculation of: (1) normal or ensheathed third-stage larvae (L3) (Cypess, 1970a; Rubin et al., 1971; Lueker and Hepler, 1975; Chaicumpa et al., 1977; Zidian *in* Cypess et al., 1977; Penttila et al., 1984); (2) adult worms (Jacobson et al., 1982; Hurley and Vadas, 1983; Mitchell and Munoz, 1983); (3) L3 antigen in Freund's complete adjuvant (Cypess, 1970b; Goven and DeBuyascher, 1980); (4) high doses of adult worm E/S antigens (Hurley et al., 1980), or high doses of lyophilized adult worm extract (Mitchell and Cruise, 1984) both in pertussigen adjuvant. (B) Oral immunizing infection(s) with L3 or irradiated L3. In order to study immunological events that are relevant to the outcome of natural infections, an oral immunizing infection with L3 larvae seems to be more appropriate than systemic inoculation of the parasite or its products. However, even when studying oral infections, a number of variables may influence the outcome of such experiments. Of particular interest to us were: (1) the effects of varying the duration of the immunizing infection and (2) the effects of varying the number of worms given in such immunizing infections.

It has been suggested that L4 larvae, developing in the wall of the small intestine, stimulate acquired immunity and that this stage may be the target of such responses in subsequent infections (Bartlett and Ball, 1974; Pritchard et al., 1984). If this is true, the duration of the immunizing infection and the size of the immunizing inoculum may be important as adult worms in the lumen of the intestine appear not to stimulate immunity (Jacobson et al., 1982) and may in fact suppress the host's response (Behnke et

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* Present address: Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721.
† Present address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, Wisconsin 53706.

al., 1983). Behnke and Robinson (1985) found that a 9-day anthelmintic-abbreviated (pyrantel embonate) immunizing infection was more effective at stimulating immunity than the longer periods of immunization. In the experiments of Behnke and Robinson, however, the anthelmintic used is not effective against larvae developing in the gut wall. Thus, worms must emerge into the lumen of the gut in order to be killed. We were curious to determine the effect of killing L4 worms *in situ* such that adult worms never emerged. In the present report we show that even highly susceptible strains of mice can be protected against challenge if a 6-day ivermectin-abbreviated primary infection is used for immunization. In addition, we show that the number of parasites administered in the primary infection can markedly influence the development of resistance to challenge.

MATERIALS AND METHODS

Animals

Female mice, 8–12-wk-old, were used in all the experiments. B10.Q, B10.BR, and B10.M mice were reared in the immunogenetics colony at Cornell University. DBA/1J and CBA/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Mice were housed, 5 each in 11⅝ × 7½ × 5-in. polycarbonate cages, fed Charles River RHM 1000 rat, mouse, and hamster formula, and given water *ad libitum*. All mice were kept on a photoperiod of 12-hr daylight and 12-hr darkness.

Parasite

Nematospiroides dubius was maintained by a modified technique of Cypess et al. (1973). *Nematospiroides dubius* third-stage larvae were obtained from eggs collected from infected B10.T(6R) female mice. The ova-containing feces of these source mice, infected with 300 L3 for no longer than 2 mo, were collected over a 24-hr period by placing moist filter paper in the bottom of a collection cage. The fecal pellets were mashed, mixed in distilled water, and filtered through 2 layers of gauze. The filtered suspension was washed by alternate centrifugation (100 *g* for 2 min) and resuspension in distilled water until the supernatant was clear. The fecal sediment was spread in a thin layer on top of 6 layers of wet filter paper in a covered 15-cm culture dish. The fecal cultures were incubated at room temperature and were aerated and moistened with aerolized distilled water each day. The larvae were collected 6–8 days later by aspiration with a Pasteur pipette after rinsing the culture dish, and were washed 5 times by alternate centrifugation (100 *g* for 2 min) and resuspension in distilled water. The larvae used for experimental infections were stored in water at 4 C for no longer than 3 wk prior to use. To prepare larvae for infections, they were resuspended in water with a stirring bar, 10 aliquots were counted and the concentration was adjusted to the desired level. The mice were infected *per os* using a [illegible] syringe (Hamilton, Reno, Nevada) equipped with a blunt-curved 18-gauge needle. The larvae, regardless of the number given, were suspended in 0.2 or 0.25 ml of water.

Anthelmintics

Ivermectin (Eqvalan MSD Agvet Inc., Barceloneta, Puerto Rico) was administered *per os* (8 mg/kg body weight) with an 18-gauge blunt-curved needle attached to a 1-ml syringe.

Experimental design

Groups of 8 mice each received primary immunizing infections with either 50, 150, or 300 L3. The infection was interrupted with ivermectin on either day 6 or day 14 postinfection, and 6 days later each mouse received a challenge infection with 100 L3. The control groups in each experiment received the anthelmintic treatment and the challenge infection the same days as the experimental groups. On day 21 postchallenge infection the mice were killed, the small intestine was removed, and beginning at the pylorus, the small intestine was torn along its longitudinal axis with forceps. The adult worms were removed and counted individually as the intestine was opened.

Analysis of results

The results of worm counts in animals receiving challenge infections are expressed as a mean percentage of counts obtained in primary infection controls; the adult worm count from each mouse was divided by the mean count for adult worms recovered from the primary infection control group and multiplied by 100. The results were analyzed for significance using the nonparametric Mann-Whitney rank sum test (Snedecor and Cochran, 1980).

RESULTS

We have shown previously that mice expressing the H-2^q or H-2^f haplotypes are resistant to challenge infections with *N. dubius* when compared to mice expressing H-2^k alleles (Enriquez et al., 1985); in these experiments mice were immunized with a 14-day, anthelmintic-interrupted *per os* infection. This regimen allows sufficient time for adult worms to emerge from the gut wall and establish in the lumen of the small intestine. Animals in which L4 larvae were killed *in situ* on day 6 (Sayles and Jacobson, 1983) were compared with animals immunized via a standard 14-day immunizing infection. Thus, one group of mice was exposed to adult worms and the other was not.

B10.Q (H-2^q), B10.M (H-2^f), and B10.BR (H-2^k) mice were each divided into 2 groups of 8 mice each. Group 1, in each case received 50 L3 on day 0, whereas mice in group 2 received 50 L3 on day 8. On day 14, all mice along with primary infection controls, were treated with ivermectin (8 mg/kg); at this time, group 1 mice

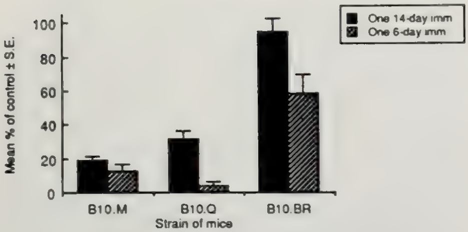

FIGURE 1. Effect of 14-day and 6-day (50 L3) ivermectin-interrupted immunizing infection on challenge with 100 L3 of *N. dubius*.

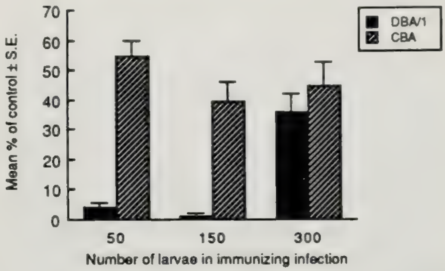

FIGURE 2. Effect of varying number of larvae during a 14-day standard immunizing infection upon challenge.

had been infected for 14 days, whereas group 2 mice had been infected only for 6. Six days following ivermectin treatment, all mice received 100 L3 and 21 days later were killed for worm counts. Results are shown in Figure 1. As expected, B10.Q and B10.M mice resisted challenge following either immunizing regimen. B10.BR mice were susceptible to challenge when compared to the resistant strains ($P < 0.05$) but were more susceptible to challenge if immunized such that adult worms were allowed to develop ($P < 0.07$). B10.BR mice developed complete resistance to challenge following 2 immunizing infections regardless of which immunizing regimen was used (data not shown).

Next, we examined the influence of parasite numbers on the development of resistance to challenge. Because very large numbers of mice were required to study the effects of both dose and immunization regimen on the outcome of infection, it was not possible to conduct these studies using age-matched, H-2 congenic strains of mice as was done in the previous experiment. Instead, we purchased DBA/1J and CBA/J mice from the Jackson Laboratory. We had shown previously that DBA/1J mice (*H-2^q^*) were very resistant to challenge when compared to CBA/J (*H-2^k^*) (Enriquez et al., 1985). Where the data in Figure 1 relate to H-2 controlled effects, data for the experiments reported below may reflect influences attributable to both H-2 and non-H-2 genes. DBA/1J and CBA/J mice were each divided into 6 groups of 8 mice each. Groups 1–3 received a standard 14-day immunizing infection, whereas groups 4–6 had infections terminated on day 6. Groups 1 and 4 were immunized with 50 L3, groups 2 and 5 received 150 L3, and groups 3 and 6 received 300 L3 in the priming infection. All mice, along with the primary infection controls were challenged with 100 L3 6 days following ivermectin treatment. Control mice that were infected at the above immunizing doses harbored 65–75% of the inoculum, and control mice that were treated with ivermectin 6 or 14 days postimmunizing infection had no worms upon necropsy. After a 14-day immunizing infection, CBA mice, regardless of the immunizing dose, harbored significant numbers of worms in the small intestine. In contrast, DBA/1J mice resisted the challenge providing that the immunizing dose was low (50 and 150 L3; $P < 0.01$ and $P < 0.05$, respectively, compared to CBA mice), but failed to resist challenge following a single immunization with 300 L3 ($P > 0.05$) (Fig. 2). The pattern of responsiveness differed markedly for mice receiving the 6-day abbreviated infection (Fig. 3); DBA/1J mice resisted challenge regardless of the infective dose. CBA/J mice, however, were not immunized following the 6-day regimen unless high doses of L3 were administered.

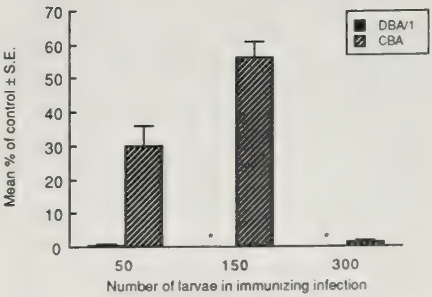

FIGURE 3. Effect of varying number of larvae during a 6-day abbreviated immunizing infection upon challenge.

DISCUSSION

Our results support previous observations that the presence of adult worms in the lumen of the small intestine interferes with the development of resistance to challenge infections with *N. dubius* (Jacobson et al., 1982; Behnke and Robinson, 1985). At low immunizing infective doses, resistant strains of mice appear to overcome this suppressive influence of luminal adult worms and an effective anti-parasite response developed. At high infective doses, however, even resistant strains of mice may be suppressed. Interestingly, if L4 larvae from a large immunizing inoculum are killed *in situ* and adult worms never emerge, even susceptible strains of mice may resist challenge. This suggests (1) that susceptible strains may be more easily suppressed than resistant strains of mice, and (2) that the susceptible phenotypes may possess the ability to mount effective anti-parasite responses but need a higher immunogenic antigen load. The dose-related effects observed in the *N. dubius* system parallel those observed in *T. spiralis*-infected mice and similar H-2 genes appear to influence susceptibility to infection in these different host–parasite systems (Wassom et al., 1984; Enriquez et al., 1985). In both cases, mice expressing *H-2k* alleles are particularly susceptible to infection/challenge. Interestingly, resistant strains of mice tend to express H-2 haplotypes characterized by lack of I-E expression. It has been proposed that recognition of relevant *N. dubius* or *T. spiralis* antigens in the context of I-E molecules induces a response that suppresses the expression of functional immunity (Wassom et al., 1987). The present results suggest that in the *N. dubius* system it is adult worm antigens or alternatively antigens presented to the immune system via transport through the intestinal mucosa that induce the suppressive response. Our results also support conclusions drawn by a number of other investigators. Behnke and Robinson (1985) observed that a 9-day abbreviated immunizing infection was very effective in eliciting protection against *N. dubius* challenge when compared to immunizing infections wherein adult worms were allowed to reside in the lumen of the intestine. In their experiments, they eliminated worms from primary infections using pyrantel. In our experiments we used ivermectin, which, in contrast to pyrantel, is effective against larval stages *in situ* (Sayles and Jacobson, 1983). The 6-day abbreviated immunizing infection was extremely effective in triggering protection to challenge. Behnke et al. (1983) suggested that *N. dubius* can suppress the immune response it engenders in mice, and proposed that this immunosuppressive effect may be mediated more rigorously by adult worms than larval stages; they demonstrated that infections with irradiated L3, which do not develop into adulthood, elicited strong immunity that was not present if normal L3 were given concurrently and developed into luminal adult worms. Surgical transplantation of adult worms into the lumen of the intestine, before or after immunizing larval infections, lowered resistance to challenge infections also suggesting a strong immunomodulatory effect of adult worms (Jacobson et al., 1982). Mice infected with *N. dubius* also exhibit nonspecific immunosuppression to T-dependent and T-independent antigens (Ali and Behnke, 1984b), and it has been demonstrated that both larval and adult stages are responsible for nonspecific immunosuppression (Ali and Behnke, 1984a).

Behnke and Robinson (1985) observed that increasing the number of larvae in a 9-day abbreviated immunizing infection enhanced the protection to challenge in a resistant strain of mouse. However, in a 21-day immunizing infection, Sitepu et al. (1985) found that increasing the number of larvae (from 5 to 80) enhanced protection to challenge in a resistant strain but suppressed a susceptible strain. In a subsequent study, Dobson et al. (1985) studied a wider range of immunizing larval doses (from 10 to 400) in outbred Quackenbush mice and observed that higher numbers of larvae in the immunizing infection resulted in higher adult worm burdens recovered at challenge. The latter observations agree with our findings, in which resistant DBA/1J mice developed resistance after a standard immunizing infection with 50 or 150 L3, but failed to do so if 300 L3 were administered.

We observed that a susceptible strain of mouse (CBA/J) could be immunized against challenge when the 6-day immunization regimen was used and high doses (300 L3) of larvae were given. Pritchard et al. (1984) demonstrated that the L4 stage of *N. dubius* possesses a stage-specific antigen (16 kDa) that was functionally immunogenic. It is possible that the 6-day abbreviated immunizing infection at high larval doses allows susceptible strains to be sensitized to this and/or other functionally relevant antigens, and that in the absence of immunomodulation by adult worms, functional immunity may develop.

Our data demonstrate that multiple factors may influence the outcome of experimental infections with antigenically complex parasites. Although the ability to resist challenge infections with *N. dubius* is genetically controlled and influenced by both H-2 and non-H-2 genes, the expression of these genes is influenced by a number of variables. Particularly significant are the stages in the parasite's life history to which the host is exposed, and the numbers of parasites present in each of these stages.

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INTESTINAL LEAKAGE AND PRECIPITATING ANTIBODIES IN THE SERUM OF QUILLBACK, *CARPIODES CYPRINUS* (LESUEUR), INFECTED WITH *NEOECHINORHYNCHUS CARPIODI* DECHTIAR, 1968 (ACANTHOCEPHALA: NEOECHINORHYNCHIDAE)

Alexander J. Szalai, G. V. Danell, and Terry A. Dick
Department of Zoology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

ABSTRACT: Leakage of the intestine of quillback, *Carpiodes cyprinus*, infected with *Neoechinorhynchus carpiodi* was examined using Evans blue (EB) injected into the bloodstream of quillback. The concentration of EB-labeled blood proteins in the intestinal lumen was greatest at the sites of nodule formation and was correlated with size of nodules and number of gravid female worms attached. The intestines of uninfected quillback with no signs of previous infection (no nodules) had the lowest level of protein leakage. Immunoprecipitation bands were observed when sera from infected fish were tested with purified *N. carpiodi* antigens. These bands were due to specific antibody reactions and not due to C-reactive protein (CRP) or alpha-migrating factor. Antibody titres were not correlated with intensity of infection but the proportion of fish testing positive increased with increasing number of worms. Precipitation reactions were most intense against mature male and gravid female worms and these shared a common antigen. The persistence of nodules and the presence of antibodies suggests that this host–parasite association involves both cellular and humoral components of the fish immune system. This ensures the integrity of nodules and the associated vascularization and leakiness that appear to be important in supplying nutrients to the parasites.

The pathology associated with infections of the acanthocephalan *Neoechinorhynchus carpiodi* Dechtiar, 1968, in quillback, *Carpiodes cyprinus* (Lesueur), has recently been described (Szalai and Dick, 1987). *Neoechinorhynchus carpiodi* elicits nodule formation at the sites of proboscis attachment and nodule numbers, size, and pathology are related to the number of worms and to the depth of proboscis penetration. When gravid female *N. carpiodi* penetrate beyond the stratum compactum, the resulting breakdown of the mucosal barrier and extensive vascularization may cause the intestine to be more leaky in the region of the nodules, thereby enhancing fluid contact between parasite and host and the possibility of developing an immune response by the host. The persistence of *N. carpiodi* and the nodules further suggests that there might be prolonged stimulation of the quillback immune system.

Little information is available on the immune response of fish to intestinal metazoan parasites (Kennedy and Walker, 1969; Harris, 1972; McArthur, 1978). Precipitating antibodies to intestinal infections of *Pomphorhynchus laevis* and *Telogaster opisthorchis* were reported by Harris (1972) and McArthur (1978), but neither of these studies considered the possibility of false positives due to C-reactive protein (CRP) or alpha-migrating factor, which have been identified in some fish sera (Baldo and Fletcher, 1973; Alexander, 1980). These proteins are nonspecific and form precipitation bands similar to specific antigen–antibody precipitin bands observed in Ouchterlony double-diffusion plates and therefore must be considered if precipitin reactions in gels are to be interpreted accurately.

The objectives of this study were to determine if (1) the pathology associated with nodules and the acanthocephalan *N. carpiodi* in the quillback intestine enhanced the movement of fluids into the gut lumen, and if (2) quillback produced precipitating antibodies to *N. carpiodi*.

MATERIALS AND METHODS

Measuring gut leakiness

Quillback were collected with live traps at Dauphin Lake (51°17′N, 99°48′W), Manitoba, Canada, during August and September 1986 and in June 1987 (water temperature 12–16 C). Length, weight, sex, and maturity of fish were noted at time of death.

Plasma protein concentration was measured using Evans blue (EB) as an indicator. Evans blue (2.0 ml, 1% solution, autoclaved) in phosphate-buffered saline (PBS) was injected into the heart of unanesthetized fish with an 18-gauge needle. Quillback were kept in a 27-m^3 enclosure at water temperatures of 12–16 C and sacrificed 12, 15, 17, or 23 hr postinjection. Fish were killed by a blow to the head. The gastrointestinal tract (from the oesophageal–intestinal junction to the anus) was removed and placed on a biased-grid wax board (Brambell, 1965) and the entire outer surface of the gut

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wiped clean with a damp sponge to avoid contamination of the lumenal contents with blood. Each intestine was divided into 3 sections: a prenodular section including all the intestine anterior to the sites of *N. carpiodi* attachment, a central nodular section where all the worms and nodules were found, and a postnodular section posterior to the nodular section. Each section was flushed with 4–8 ml of PBS and then gently squeezed to remove any lumenal contents. All helminths recovered from the intestines were enumerated, identified, and recorded.

The lumenal contents of each section were homogenized using a 10-ml ground glass tissue homogenizer and the homogenate adjusted to 12 ml with PBS. A 5-ml aliquot of the diluted homogenate was mixed with an equal volume of 10% trichloroacetic acid (TCA) to test if EB was bound to protein and not present as unbound dye. Formation of a blue precipitate indicated the absence of unbound dye (Nawa, 1979). Serum (0.075 ml) was also tested with TCA to check that EB had been successfully injected. Quillback that were improperly injected had sera that did not produce a blue precipitate and were not used in the analyses.

Four ml of acetone (−10 C) was added to an equal volume of diluted homogenate to extract protein-bound EB and to remove turbidity (Allen, 1951). The solution was mixed and then centrifuged for 8 min at 7,000 rpm in a Model CL clinical centrifuge (International Equipment Co., Needham HTS., Massachusetts). The optical density (OD) of the resulting supernatant was immediately measured at 620 nm using a Bausch & Lomb Spectronic 20 spectrophotometer and compared to a PBS-acetone blank.

Blood samples (2–6 ml/fish) collected from the ventral aorta by cardiac puncture were left approximately 30 min at room temperature and then placed at 4 C and allowed to coagulate for at least 4 hr. These samples were then centrifuged (2 min at 7,000 rpm) and the serum collected. Serum samples (0.15 ml) were diluted 100 times with PBS before the OD was measured.

A standard curve for EB concentrations was prepared by mixing 4 ml of EB-PBS solution (serial dilutions of from 10 to 4×10^{-5} g/ml EB) with 4 ml acetone, and absorbance was measured at 620 nm (Bradford, 1976). The OD recorded for each sample was transformed into corresponding measures of EB concentration (CEB) for lumenal (CEBL) or serum (CEBS) contents using the standard curve. Because the CEB values were determined from samples previously diluted with PBS and acetone, an estimate of undiluted CEB (uCEB) was made, where uCEBL = CEBL × 2, and uCEBS = CEBS × 200.

Six uninjected fish were our controls and these were used to determine the levels of background absorbance at 620 nm from the lumenal contents. A correction for background absorbance was determined using the equation CEBLc = uCEBL − B, where CEBLc is the concentration of EB after adjusting for the dilution factor and background absorbance, and B is the average uCEBL value attributable to background absorbance. A further calculation was used because prenodular, nodular, and postnodular sections of the gut were not always of equal length. All CEBLc values were divided by the length (cm) of the section and recorded as CEBLc/cm of intestine to permit comparison between sections of gut in the same fish and among sections from different fish.

The concentration of EB in the intestine (LI) was determined using LI = ([CEBLc/uCEBS]/cm), where LI is the ratio of the concentration of EB in lumen (after corrections) versus serum contents.

After the lumenal contents had been collected, the open ends of each gut section were clamped to prevent loss of worms. These sections were kept at 4 C and examined within 24 hr of host death by immersing in water and slitting longitudinally. Location and number of all *N. carpiodi* and/or nodules were recorded, and sex and maturity of all worms noted. Worms were removed, washed twice in PBS, and stored at −80 C. Each nodule was measured for maximum width (MW), maximum length (L), and maximum depth (D), and normal gut thickness (T) was measured 2 cm from the edge of the nearest nodule.

The volume of each nodule was determined as previously (Szalai and Dick, 1987) using the formula for one-half of an ellipsoid; $\frac{1}{2}\pi LW^2$, where $W = (MW + [D - T])/2$.

Testing for antibody

Antigens were prepared from immature and mature male and gravid and nongravid female *N. carpiodi* that had been stored at −80 C. Worms were thawed and immediately homogenized in 5 ml of PBS until microscopic examination of the suspension revealed no large particles. Homogenates were centrifuged at 17,000 rpm for 30 min at 4 C in a Beckman J2-21 centrifuge, and the supernatants pipetted into dialysis tubing (12,000–14,000 molecular weight exclusion/ Spectropore, Spectrum Medical Industries Inc., Los Angeles) and dialysed for 48 hr against PBS (4 changes of 1 L each). Solutions containing purified antigens were collected after dialysis and centrifuged as above. Aliquots (0.5 ml) of purified antigen were stored at −80 C. Protein determinations were made on each aliquot using the micromethod of Lowry et al. (1951).

Blood samples were collected from 61 quillback, and undiluted serum was isolated and stored in 1.5-ml microcentrifuge tubes at −80 C until needed. Five uninfected individuals showing no signs of previous infection (no nodules) served as our controls. Serum was tested for precipitating antibody against antigens of *N. carpiodi* using a modification of the Ouchterlony double-immunodiffusion technique (Ouchterlony and Nilsson, 1973). Samples were tested in gels (1% agarose : 3% polyethylene glycol-6000) made up in 0.1 M tris(hydroxymethyl)aminoethane (Tris) buffer at pH 8.3.

After diffusion the gels were rinsed (30 min) in 5% sodium citrate, washed (12 hr) in 2% saline and distilled water (1 hr), air dried, and stained with 1% amido Schwartz 10B (Keleti and Lederer, 1974).

To ensure that precipitin bands were due to the presence of specific antibody and not due to CRP or alpha-migrating factors, 0.1 M disodium ethylenediaminetetracetic acid (EDTA) was incorporated into the gel buffer to block any nonspecific precipitation reactions (Ellis, 1985). Serial dilutions of fish sera and worm antigens were tested for possible relationships between antibody titre and number of worms per fish. Antibody titre was taken as the reciprocal of the highest antigen

dilution giving visible precipitation with undiluted antibody.

Statistical analysis

Regression equations were derived using simple linear and polynominal regression. Concentration values were log transformed prior to analysis. Each section of intestine was placed into 1 of 2 categories based on log LI values, and stepwise discriminant analysis (Bennet and Bowers, 1976) was applied to choose independent variables that best predicted the assignment of intestinal sections to each category. Quillback were put into 1 of 2 classes based on the number of *N. carpiodi* they harbored, and standardized measures of skewness based upon the third moment around the mean (G_1) (Bennet and Bowers, 1976) were calculated to compare the frequency distributions of titres for positive sera from fish with low or high numbers of worms. A probability of $P < 0.05$ was considered significant in all tests. Analyses were performed using the Statistical Analysis Systems package (SAS Institute, Inc., Box 8000, Cary, North Carolina, 1982 edition) as implemented by the University of Manitoba Computer Services.

RESULTS

Concentration of EB in the intestine

Sixty-one quillback were treated with EB and 60 of these were infected with *N. carpiodi.* The extent of normal intestinal leakiness was determined from the remaining fish whose intestine showed no evidence of previous infection by *N. carpiodi.* Injection of EB into the uninfected quillback showed that little protein-bound EB passed from the body to the lumen of the intestine. The concentration of EB in the uninfected quillback intestine (log LI = −4.25) was outside the 99% confidence limits for infected quillback prenodular (log LI = −2.24 to −2.99), nodular (log LI = −0.23 to −1.25), and postnodular (log LI = −2.19 to −2.94) regions. Eight of the quillback treated with EB harbored *Lissorchis gullaris* (3.3 ± 2.6 worms per infected fish), 11 had *Rowardleus pennensis* (10.9 ± 14.4), and 3 had *Monobothrium hunteri* (2.3 ± 1.2). Pathology was not observed in quillback without *N. carpiodi. Neoechinorhynchus carpiodi* were not recovered when the intestines were flushed with PBS and were firmly attached and alive when nodules were examined 24 hr after collection.

There was no significant difference in the concentration of EB (LI) in the 3 regions of intestine at 12, 15, 17, and 23 hr postinjection, although the amount of EB in the nodular sections was highest (log LI = 1.11 ± 2.36) at 15 hr postinjection. The concentration of EB in the lumen of the intestine was greatest in the section containing the nodules and in slightly higher concentrations in the postnodular region than in the prenodular region (Fig. 1).

Sections of intestine were classified as having high (log LI ≥ −1.62) or low (log LI ≤ −1.61) concentrations of EB and this was compared to the total volume of nodules. A stepwise discriminant analysis based on 5 variables (time postinjection, length, site of worm attachment, number of worms per fish, and number of nodules per fish) was done. The discriminant procedure chose number of nodules as the best variable for discriminating between intestinal sections. Eighty percent of the intestinal sections could be correctly classified based on the number of nodules present and the number of nodules was correlated ($r^2 = 0.64$) with the number of gravid female worms.

Serum tests

The protein concentrations of the undiluted antigens were: gravid females, 5.8 mg/ml; mature male, 4.2 mg/ml.

Precipitin bands (Fig. 2) were observed in 33 of 61 (54%) sera tested (titre = 8.12 ± 12.36), and the distribution of positive sera was skewed toward high titres ($G_1 = 3.199$). Fourty-two fish had low numbers of *N. carpiodi* (0–40 worms per fish) and only 40% of these tested positive. Nineteen fish had high numbers of worms (≥41 worms per fish) and 84% were positive for *N. carpiodi* antibodies. The titre for fish with low numbers of worms (7.88 ± 9.78) was not significantly different from that for fish with high numbers of worms (8.38 ± 15.31) (*t*-test), but the distribution of positive sera was more skewed for fish with high numbers of worms ($G_1 = 2.959$) than for fish with fewer worms ($G_1 = 1.689$).

Four of five sera from fish with no worms but showing evidence of previous infection (nodules) tested positive (titre = 3.0 ± 1.15). Sera from 4 fish known to be uninfected and with no nodules or other signs of prevous infection by *N. carpiodi* all tested negative. One sample of serum from an uninfected quillback with no nodules gave a weak positive response (titre = 2).

The proportion of positive sera increased linearly with intensity of infection ($r^2 = 0.75$). However, the proportion of positive sera with high (≥4) titres reached a maximum for fish with 21–40 worms, and decreased for fish with more worms (Fig. 3).

Seven of 21 sera tested in a separate experiment gave false positives (titre = 3.14 ± 2.27)

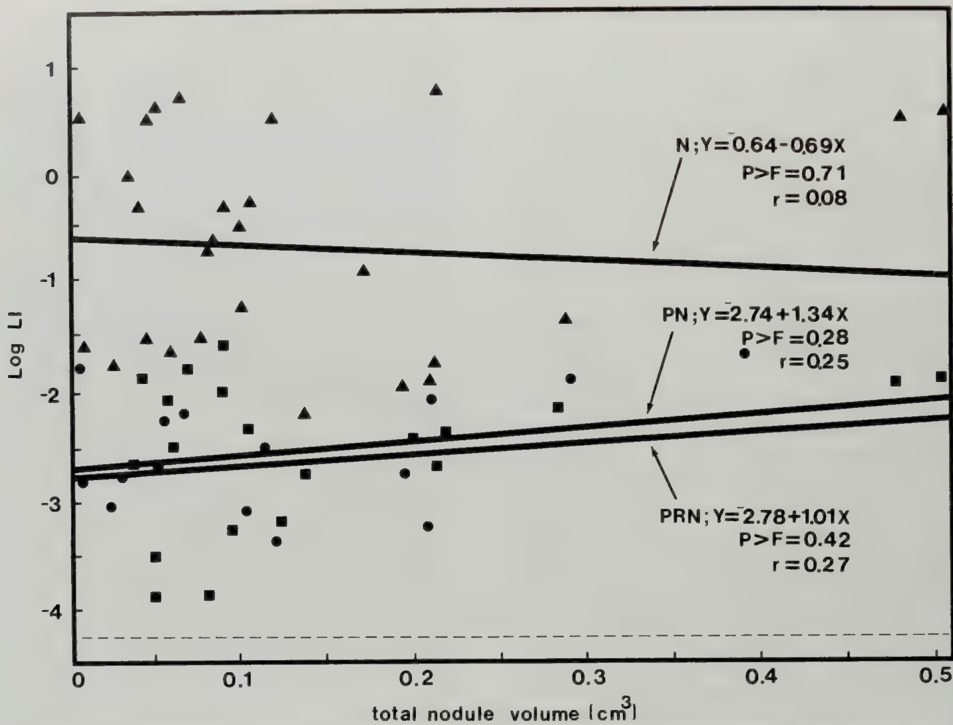

FIGURE 1. Relationship between the concentration of protein-bound Evans blue (log LI) in the lumen and the total volume of nodules (cm^3) in the intestine for *N. carpiodi*-infected quillback. Regression equations for concentration of bound EB versus volume are given and plotted separately for prenodular (PRN, ●), nodular (N, ▲), and postnodular (PN, ■) regions. Dashed line indicates the level of protein-bound EB in the lumen of an uninfected (nodule-free) quillback.

in gels not containing EDTA; the remaining sera (n = 14) gave responses in EDTA-treated gels that were lower than or equal to the responses in untreated gels. The effectiveness of EDTA treatment on reducing nonspecific precipitation was further confirmed in tests where sera from different quillback were diffused against each other. Strong precipitation reactions occurred between one pair of sera but these were absent in EDTA-treated gels.

DISCUSSION

Our study has clearly shown that the intestine of quillback infected with the acanthocephalan *N. carpiodi* is leaky to large proteins from the blood. This leakiness, expressed through the complexing of EB with serum proteins, is most pronounced in the region of nodules induced by *N. carpiodi*. The EB measured in the intestine of quillback is due primarily to leakage into the lumen, where breakdown of the mucosal–tissue barrier occurred, because uninfected quillback showed extremely low levels of EB in the absence of any such pathology. The concentration of EB in the lumen of the gut of uninfected quillback was much lower than that recorded from the anterior and posterior sections of infected quillback intestines, and this difference was most pronounced where nodules were present. Increasing concentrations of EB in the intestine of quillback were correlated with increasing numbers of worms and nodules which further supports the idea that gut leakiness in quillback is largely due to the presence of *N. carpiodi* and associated pathology.

The leaky nature of the intestine in the region of the nodules indicates that large molecules, including antigens, could cross the mucosal–tissue barrier. Indirect evidence for this is shown through an immune response in the form of precipitating antibodies to *N. carpiodi* antigens. Furthermore, there is no doubt that this response

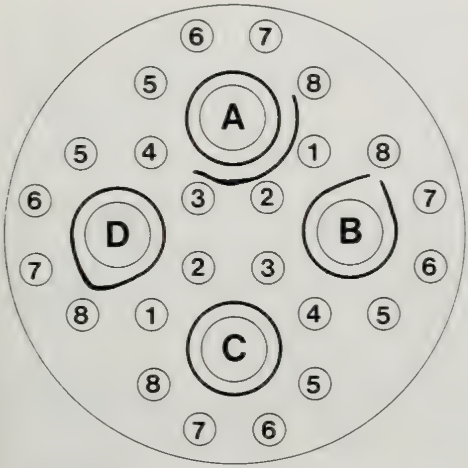

FIGURE 2. Precipitin bands resulting from double immunodiffusion of quillback serum against *N. carpiodi* antigens. A–D. Large wells containing quillback serum (A, undiluted serum; B, 50% serum; C, 25% serum; D, 12.5% serum). 1–4. Small wells containing gravid female *N. carpiodi* antigens (1, undiluted antigen; 2, 50% antigen; 3, 25% antigen; 4, 12.5% antigen). 5–8. Wells containing mature male antigens (5, undiluted antigen; 6, 50% antigen; 7, 25% antigen; 8, 12.5% antigen).

involves specific antibody–antigen reactions, because we ruled out the effects of nonspecific reactions due to CRP and alpha-migrating factor. Whether host tissue needs to be damaged to allow parasite antigens access to the humoral system (Smyth, 1969; Harris, 1972) or whether such antigens can be transported through the intact epithelium (Rombout et al., 1985) is not known. Rees (1967) suggested that immune responses to intestinal fish parasites will develop only if the host mucosa is invaded. Harris (1972) found specific anti-worm antibody in the intestinal mucosa of chubs infected with the acanthocephalan *Pomphorhynchus laevis* and concluded that nonspecific leakage of precipitins from the blood to the mucus might be due to associated tissue damage. In our study there was a strong immune response to antigens derived from male and female acanthocephalans and it is likely, in natural infections of quillback, that *N. carpiodi* antigens reach the bloodstream via these parasite-induced lesions. Whatever the mechanism of antigen presentation, a continued but low-level stimulation of the quillback immune system seems likely, because the intensity and distribution of *N. carpiodi* in-

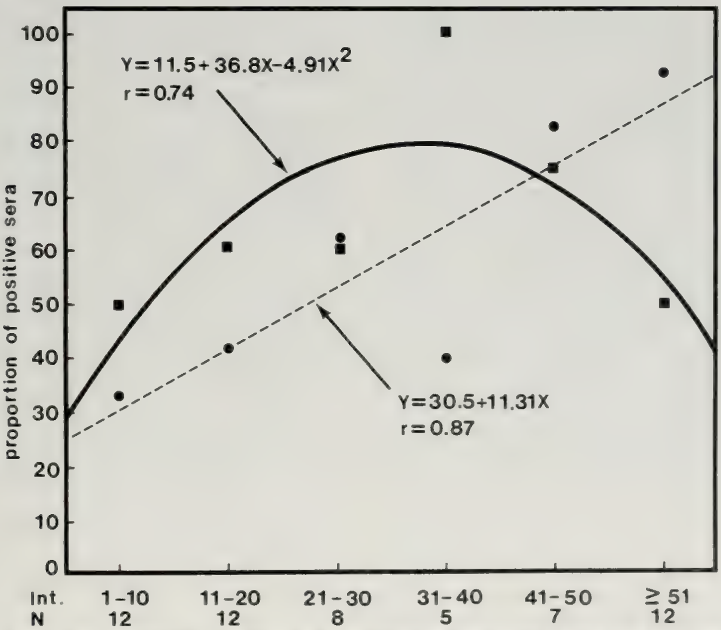

FIGURE 3. Relationship between the number of *N. carpiodi* per quillback (Int.) and (1) the proportion of positive sera (●) and (2) the proportion of positive sera with a titre >4 (■). Regression equations for positive sera (dashed line) and for positive sera with a titre >4 (solid line) are given. N: number of fish per class.

fections were not affected by host sex, size, or the season of capture (Szalai and Dick, 1987). Furthermore, fish with the highest intensity infections had lower titres and this suggests the possibility of immunosuppression.

It appears that in quillback infected with *N. carpiodi* the antibody response is persistent and due to a continuous stimulation of the host immune system. Furthermore, because vascularization appears to play an important role by bringing blood fluids next to the only break in the mucosal–tissue barrier, the persistence of the nodules and the limited area and leaky nature of the lesions might ensure that a limited but steady supply of nutrients is available to the parasites.

This host–parasite system, because of the persistent humoral and cellular responses manifested in nodule formation and the possibility of immunosuppression, has interesting possibilities for understanding the roles of the cellular and humoral components of the immune system of fish.

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IN VITRO CULTIVATION OF *HERPETOSOMA* TRYPANOSOMES ON EMBRYONIC FIBROBLASTS AND IN SEMIDEFINED CELL-FREE MEDIUM

H. A. Mohamed, S. Maraghi, K. R. Wallbanks, and D. H. Molyneux
Department of Biological Sciences, University of Salford, Salford, United Kingdom M5 4WT

ABSTRACT: *Microtus agrestis* embryo fibroblasts (MAEF) support the survival and multiplication at 37 C of the mammalian multiplicative forms of the *Herpetosoma* trypanosomes *Trypanosoma microti*, *T. evotomys*, *T. musculi*, and *T. lewisi* passaged from cultures on Schneider's *Drosophila* medium and of *T. grosi* from Grace's medium. MAEF layers with parasites were maintained for a period of over 5 mo.

A semidefined cell-free medium also supported the multiplication (at 37 C) of the mammalian forms of the same trypanosome species, passaged directly from Schneider's *Drosophila* medium or Grace's medium, without their prior culture on cell lines.

Reproductive stages were observed in cultures; *T. microti* and *T. evotomys* produced nests of dividing amastigotes from which trypomastigotes developed in the medium supernatant. *Trypanosoma lewisi*, *T. musculi*, and *T. grosi* divided initially as epimastigotes, which then transformed to bloodstream trypomastigotes. Multiplication of trypomastigotes was also observed. These methods of reproduction are the same as those reported in the respective mammalian hosts.

Several investigators have cultivated *Herpetosoma* trypanosomes in association with various mammalian cell lines (see review by Molyneux, 1976). Table I summarizes the attempts that have been undertaken to date. Several of these studies predated the successful culture of the pathogenic salivarian trypanosomes with mammalian cell lines achieved by Hirumi et al. (1977a, 1977b) for *T. brucei*, Gray et al. (1981) for *T. congolense*, and Brun and Moloo (1982) for *T. vivax*.

In this study, *T. microti*, *T. evotomys*, *T. musculi*, and *T. lewisi* were transferred from cultures in Schneider's *Drosophila* medium and *T. grosi* from Grace's medium (Mohamed and Molyneux, 1987) to be cultivated on *Microtus agrestis* embryo fibroblasts (MAEF) and in a semidefined cell-free medium at 37 C (Baltz et al., 1985). The objective was to study MAEF feeder layers and the semidefined medium (Baltz et al., 1985) in relation to the production of the reproductive and bloodstream trypomastigote forms of *Herpetosoma* trypanosomes.

MATERIALS AND METHODS

Isolation of feeder-layer fibroblasts

The embryonic fibroblasts were derived from laboratory-bred field voles (*Microtus agrestis*) that were 14–17 days pregnant. After the animals were killed by cervical dislocation, the intact uterus was removed and washed with Earle's balanced salt solution—EBSS (Ca^{++}/Mg^{++}-free) before opening and dissecting out embryos. *Microtus agrestis* embryos were removed aseptically from the uterus, washed 3 times with EBSS solution (Ca^{++}/Mg^{++}-free), chopped into small pieces (after removing the liver from the larger embryos), incubated in 0.5% trypsin/EDTA (10×) GIBCO Biocult at 37 C, and stirred for 20–30 min. After this, the fibroblasts were isolated from the embryos using the procedure described by Brun et al. (1981).

Finally, the cells were suspended in a culture medium (RPMI-1640 with 25 mM HEPES) supplemented with 1 g/L D(+)-glucose, 10% (v/v) heat-inactivated foetal calf serum (HI-FCS), and 500 μg/ml kanamycin and distributed into T-25 (5-ml cultures) and T-75 (15-ml cultures) tissue culture flasks (Corning) to give cell densities between 5×10^6/ml and 1.5×10^7/ml and incubated at 37 C.

After 24 hr and 48 hr the medium was replaced to remove the debris and unattached cells. Within 4–6 days, depending on the growth rate of the cells, when cells were confluent, the primary cultures were trypsinized (0.05% trypsin/EDTA [1×] diluted 1:4 with EBSS [Ca^{++}/Mg^{++}-free]) and subcultured. The fibroblasts (MAEF) were cryopreserved (7.5% DMSO or 10% glycerol in RPMI-1640 with 10% HI-FCS) in liquid nitrogen after 2 subsequent subcultures.

Maintenance of fibroblast cell lines

Cultures were maintained in medium RPMI-1640 with 25 mM HEPES, 1 g/L D(+)-glucose, 10% (v/v) HI-FCS with 500 μg/ml kanamycin, incubated at 37 C, and subcultured once a week at a split ratio of 1:2.

Semidefined medium

This was described earlier by Baltz et al. (1985) and consisted of: minimum essential medium (MEM) powder (for 1 L) with Earle's salts, without sodium bicarbonate, with L-glutamine; 10 ml MEM nonessential amino acid concentrate (100×); 5.95 g HEPES; 1 g glucose; 2.2 g sodium bicarbonate; 1,100 ml double distilled water, finally supplemented with 0.2 mM 2-mercaptoethanol and 2 mM sodium pyruvate.

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TABLE I. *Summary of* Herpetosoma *trypanosomes grown in cell lines.*

Species	Author(s)	Cell line	Duration and passage no. if recorded*	Infectivity to mammals*
Trypanosoma lewisi	Hsu et al. (1968)	*Aedes aegypti* *Antherea eucalypti*	35 days/5 passages	+
	Greenblatt and Troyler (1971)	Rat spleen cells	27 days	†
	Cunningham (1977)	*Glossina* pupal gut cells	5 days	+
	Dougherty et al. (1972)	Rat kidney cells	4 mo	+
	El-On and Greenblatt (1977)	Rat lung cells	37 days	+
	Dover et al. (1983)	Chick embryos	6 days	†
Trypanosoma musculi	Cunningham (1977)	*Glossina* pupal gut cells	5 days	+
	Viens et al. (1977)	Murine peritoneal and HELA cells	23 days	+
	Albright and Albright (1978)	Mice spleen cells	8 days	†
	Clayton (1980)	Mice spleen cells	†	†
	Dover et al. (1983)	Chick embryos	−/15 passages	+
Trypanosoma rabinowitschae	Hommel and Miltgen (1974)	Baby hamster kidney cells	†	+
Trypanosoma evotomys	Hommel (1975, 1977)	Baby hamster kidney cells	†	+
Trypanosoma blanchardi	Hommel and Robertson (1976)	Baby hamster kidney cells	†	†
	Hommel (1977)	Baby hamster kidney cells	†	†
Trypanosoma grosi	Hommel (1977)	Mos 60 cells (*Aedes flaviscutella* cells)	†	†
Trypanosoma acomys	El-On et al. (1977)	*Acomys* lung cells	45 days	+

* Not reported (†); infective to mammals (+).

Trypanosomes

Primary isolation and cultivation: The primary isolation of the bloodstream forms, the origins of the parasites, and the cultivation of the vector forms of *T. microti, T. evotomys, T. musculi, T. lewisi,* and *T. grosi* were reported by Mohamed and Molyneux (1987).

Cultivation of trypanosomes on MAEF feeder layers: The overlays from *T. microti, T. evotomys, T. musculi,* and *T. lewisi* cultures in Schneider's *Drosophila* medium and from *T. grosi* in Grace's medium containing predominantly metacyclic trypanosomes (from cultures about 3 wk old) were inoculated (0.05–0.1 ml containing 4×10^4 to 2×10^5 flagellates) onto MAEF feeder layers. The MAEF were grown in their normal culture medium with the HI-FCS increased to the 20% level as used for the cultivation of trypanosomes.

The optimum age for the feeder layers to be inoculated with trypanosomes was 3–4 days after trypsinization (from the third or later subcultures), when the cells had just attained confluency. Two days after inoculation of the parasites, when the trypanosome population had become established in the MAEF feeder layers, about 50% of the overlay medium was replaced. The medium was later replaced daily and cultures were incubated at 37 C and examined daily with an inverted phase-contrast microscope.

Trypanosomes were also added to 3-day-old MAEF feeder layers in 4-chambered tissue culture slides (Lab-Tek) to study trypanosome division and the location of intercellular trypanosomes, *in situ,* after staining the slides.

Cultivation of trypanosomes in the semidefined cell-free medium: Trypanosoma microti, T. evotomys, T. musculi, and *T. lewisi* from 3-wk-old cultures in Schneider's *Drosophila* medium and *T. grosi* in Grace's culture medium were inoculated into 24-well plates (Flow Laboratories) each containing 2 ml of the semidefined cell-free medium (Baltz et al., 1985). Normally, 0.05–0.1 ml of parasite suspension was added per well and half of the medium was replaced daily. Cultures were incubated at 37 C in a CO_2 incubator (4% CO_2–96% air) and were examined daily with an inverted phase-contrast microscope.

Infectivity

The infectivity of the trypanosomes from MAEF feeder-layer cultures and semidefined cell-free medium was checked by intraperitoneal inoculation of 300–1,000 parasites of each species into their respective hosts; for *T. microti* field voles (*Microtus agrestis*), *T. evotomys* bank voles (*Clethrionomys glareolus*), *T. musculi* mice (BK/SWR), *T. lewisi* rats (BK), and *T. grosi* field mice (*Apodemus sylvaticus*). All animals were laboratory bred. All animals were examined daily for the presence of trypanosomes by wet blood smears of tail blood.

Staining

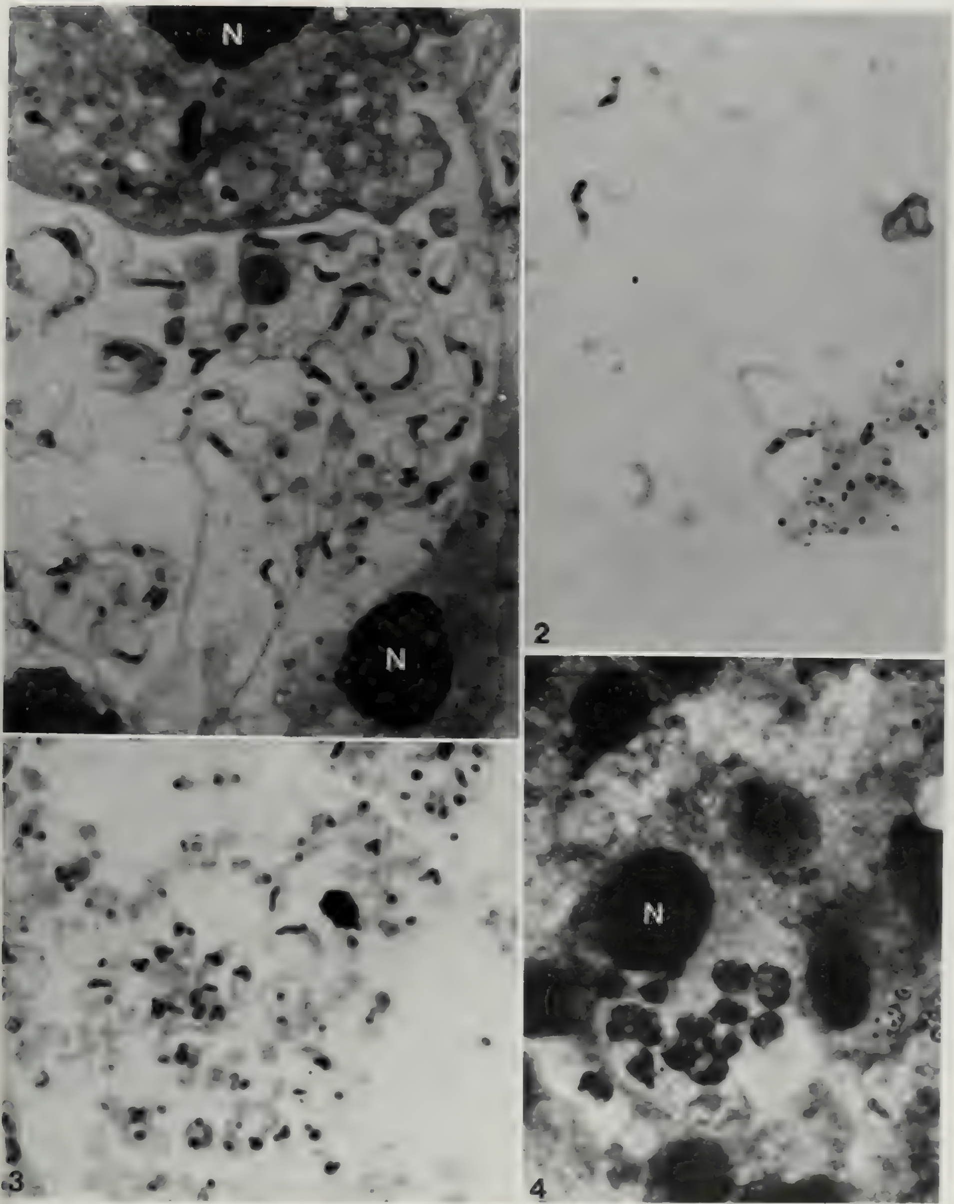
Methods of staining of parasites were described by Mohamed and Molyneux (1987).

RESULTS

Microtus agrestis embryo fibroblasts (MAEF)

Microtus agrestis embryo fibroblasts (MAEF) grew well *in vitro* and formed confluent cell layers within 4–6 days in primary culture and 5–7 days in the subcultures.

The MAEF feeder layers supported the multiplication of *T. microti, T. evotomys, T. musculi, T. lewisi,* and *T. grosi* for a period of over 5 mo. Trypanosomes inoculated onto feeder layers were observed moving between the cells within a few hours of inoculation of cell cultures. Within 1–



FIGURES 1–4. **1.** *Trypanosoma microti* trypomastigotes in between cells of a MAEF feeder layer on the third day of culture. The fibroblast nuclei (N) are prominent. Scale bar = 5 μm. **2, 3.** *Trypanosoma microti* trypomastigotes (Fig. 2) and amastigotes (Fig. 3) from the supernatant of a 12-day culture. **4.** Dividing amastigotes of *T. evotomys* in close association with fibroblasts.

2 days, groups of dividing forms were observable in the intercellular spaces and in close association with feeder layers (Fig. 1). The number of dividing and trypomastigote forms increased steadily from the third day following inoculation and these parasites formed aggregates in between the feeder layers. Many trypomastigotes were also seen growing in the supernatant (Fig. 2).

In cultures of *T. microti* and *T. evotomys*, some of the dividing forms were amastigotes (Figs. 3, 4), whereas in *T. musculi*, *T. lewisi*, and *T. grosi* the dividing forms were mostly epimastigotes. Clusters of dividing parasites were observed in all trypanosome stocks cultivated on MAEF layers.

Trypomastigotes resembling bloodstream forms were produced in the MAEF layers and grew both in the intercellular spaces and in the supernatant (Figs. 1, 2). *Trypanosoma microti*, *T. evotomys*, *T. musculi*, *T. lewisi*, and *T. grosi* trypomastigotes were infective to field voles (*Microtus agrestis*), bank voles (*Clethrionomys glareolus*), mice, rats, and field mice (*Apodemus sylvaticus*), respectively; all inoculated animals became infected. Prepatent periods ranged from 2 to 7 days.

Cultures were usually subpassaged once every month and could be successfully maintained on the same MAEF feeder layers for 2 mo by daily replacement of medium. In cultures maintained for over 4 days without medium change, the trypanosomes settled in the spaces between the cells and established clusters of various sizes on the surface of the culture flasks and fibroblasts.

Semidefined cell-free medium

The culture forms of *T. microti*, *T. evotomys*, *T. musculi*, *T. lewisi*, and *T. grosi* added to the semidefined cell-free medium began multiplication within 3–4 hr. Maximum increase in parasite numbers occurred on the second day of cultivation without a medium change or supplementation, by the fourth day, however, most of the trypanosomes were dead. If cultures were transferred to fresh medium on the second or third day of culture, they continued growth for a further 3–4 days. The daily addition of fresh medium to the cultures prolonged the duration of growth for 1–2 days.

All trypanosome stocks divided as rosettes in the supernatant and transformation into bloodstream trypomastigotes occurred within 24 hr. Trypanosomes in the supernatant were infective to their respective hosts when inoculated intraperitoneally; again all homologous recipient hosts developed patent infections after 2–7 days.

DISCUSSION

Mammalian cell lines have played an important role in the culture of pathogenic salivarian trypanosomes of the subgenera *Trypanosoma*, *Duttonella*, and *Nannomonas*; the incorporation of fibroblast feeder layers has been found to be essential for the cultivation of the bloodstream forms of these trypanosomes (Hirumi et al., 1977a, 1977b, 1983; Hill et al., 1978; Tanner, 1980; Brun et al., 1981, 1984; Brun and Moloo, 1982; Hirumi and Hirumi, 1984).

Microtus agrestis embryonic fibroblast (MAEF) feeder layers in combination with 20% HI-FCS, and RPMI-1640 medium (with 25 mM HEPES, 1 g/L D(+)-glucose, and 500 μg/ml kanamycin) supported the multiplication of *T. microti*, *T. evotomys*, *T. musculi*, *T. lewisi*, and *T. grosi* reproductive and trypomastigote forms.

The dividing forms and intercellular *Herpetosoma* trypanosomes in the feeder layers provided a continuous source of the parasites for the overlay population as the overlay (with the trypanosomes in the supernatant) was changed daily. Tanner (1980) and Brun et al. (1981) suggested that an intercellular population of *Trypanzoon* parasites was necessary for the continuous growth of cultures and that this was the major criterion that determined establishment of such *in vitro* cultures. Tanner (1980) also asserted that a short-term interaction between *T. brucei* and fibroblasts was obligatory, as the growth of the bloodstream forms ceased whenever trypanosomes were separated from the feeder-layer cells. The high concentration of trypanosomes seen in this study amongst the cells emphasizes the importance of the interaction between the parasites and fibroblasts.

The dividing forms seen on MAEF feeder layers and in the semidefined medium were morphologically similar to forms seen in the mammalian hosts of the parasites. *Trypanosoma microti* and *T. evotomys* were observed to multiply as amastigote forms both in MAEF layers (present study) and in the lymphoid tissue of *Microtus agrestis* and *Clethrionomys glareolus*, respectively (Molyneux, 1969, 1976). *Trypanosoma musculi* and *T. lewisi* multiplied as epimastigotes on feeder layers (present study) and in the blood of mice and rats, respectively, by binary or multiple fission (Galliard, 1934; Taliaferro and Pavlinova, 1936).

Baltz et al. (1985) were the first to grow the animal-infective forms of *T. brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense*, and *T. gambiense* in a semidefined cell-free medium, at 37 C. They initiated the cultures on macrophage feeder layers with the semidefined medium. After adaptation the various trypanosome stocks were subpassaged into the semidefined cell-free medium

and subpassaged every 1–3 days continuously for over 4 mo. Duszenko et al. (1985) also recently succeeded in culturing *T. brucei* bloodstream forms, at 37 C, in a cell-free medium of a modified minimum essential medium (MEM) supplemented, twice a day, with freshly prepared L-cysteine, in appropriate concentrations depending on cell density. They found that the population doubling time for trypanosomes in this cultivation system was about 12 hr compared to about 7 hr for the same clone in a feeder-layer culture system.

This need of parasites for a period of preadaptation on feeder layers or for L-cysteine, in order for them to grow in cell-free cultures at 35–37 C, may explain the short life span (4–5 days) of *T. microti, T. evotomys, T. musucli, T. lewisi,* and *T. grosi* cultures in the semidefined cell-free medium. Tissue culture cells could provide some essential nutrient for the trypanosomes or could be removing some growth-inhibiting toxic by-product from the medium which the trypanosomes produce. Alternatively, they could be an absolute requirement for the initiation of the culture, as the trypanosomes are frequently seen amongst tissue culture cells, particularly during the early development of the cultures (Hill and Hirumi, 1983).

In this study, the use of *Microtus agrestis* embryo fibroblasts for the *in vitro* maintenance and multiplication of *T. microti, T. evotomys, T. musculi, T. lewisi,* and *T. grosi* mammalian forms is recorded for the first time as is the cultivation of bloodstream trypomastigotes of the same species at 37 C without initial passage in mammalian cell lines.

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LIFE CYCLE OF *SARCOPTES SCABIEI* VAR. *CANIS*

L. G. Arlian and D. L. Vyszenski-Moher
Department of Biological Sciences and Department of Physiology and Biophysics, Wright State University, Dayton, Ohio 45435

ABSTRACT: The life cycle of *Sarcoptes scabiei* var. *canis* was systematically investigated *in vivo*. The life cycle of females and males consisted of an egg, larva, protonymph, and a tritonymph that gave rise to an adult. Development from egg to adult required 10.06–13.16 days for the male and 9.93–13.03 days for the female. Egg incubation times were >50.1 to <52.97 hr. Larval duration was between 3.22 and 4.20 days. The durations of protonymphal stages that were destined to become females and males were >2.40 to <3.40 days and >2.33 to <3.33 days, respectively. Tritonymphs destined to become females and males molted in >2.22 to <3.22 days and >2.42 to <3.42 days, respectively. During development, all life stages frequently left their burrows and wandered on the skin surface.

Sarcoptes scabiei is a common ectoparasitic mite of humans and other mammals. Periodic epidemics in communities, nursing homes, schools, and other institutions or epizootics in wild and domestic animal populations are frequently reported (Haydon and Caplan, 1971; Stone et al., 1972; Bernstein and Mihan, 1973; Blumenthal et al., 1976; Meierhenry and Clausen, 1977; Orkin and Maibach, 1978; Haarlov and Moller-Madsen, 1982; Pence et al., 1983). Accurate knowledge of the biology of *S. scabiei* is essential to understanding both the host–parasite relationship, and the epidemiology of the disease. Recently, some aspects of host specificity, the host–parasite energetic relationships, mite antigenicity and the host immune response, mite nutrition and water balance, and mite survivorship have been reported (Arlian et al., 1984a, 1984b, 1984c, 1985, 1988a, 1988b, 1988c; Arlian, 1988). However, little is directly known about other aspects of the biology and, in particular, the life cycle of this important mite species or strain. Current knowledge of the life cycle of *Sarcoptes scabiei* is primarily based upon pre-World War II observations, anecdotal accounts, or speculation about primarily human scabies, *S. scabiei* var. *hominis* (Munro, 1919; Freidman, 1947; Van Neste et al., 1981). The information is very incomplete and in some cases contradictory or incorrect. Therefore, the purpose of this study was to systematically investigate the life cycle of *S. scabiei*. Because of the taxonomic uncertainty of *Sarcoptes* strains and because the mites studied in this investigation originated from infested dogs, we will refer to them as *S. scabiei* var. *canis*.

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MATERIALS AND METHODS

Sarcoptes scabiei var. *canis* used for these studies were obtained from New Zealand white rabbits experimentally infested with *S. scabiei* var. *canis* that originated from naturally infested dogs. Infestation of the rabbits with var. *canis* was as previously described (Arlian et al., 1984b, 1984c). Heavily infested crusts from parasitized rabbits were placed in petri dishes, and the mites were first allowed to crawl from the crusts onto the dish surface and then held 24–48 hr at 10 C and 100% RH before use. Previous studies indicated that partial mite dehydration and fasting resulted in rapid burrowing when the mites were placed back on a host (Arlian et al., 1984a). Storage at 10 C did not affect the length of any life stage when compared to specimens that continuously developed on the host or when compared to fresh specimens. Therefore, because of the more rapid penetration, 24- and 48-hr-old refrigerated mites were used for some tests.

Under direct observation with a stereoscope, all active life stages were individually transferred to the inner ears (previously shaved, washed, and dried) of mite-free rabbits and allowed to penetrate. To confine the mites on the ears until they burrowed, they were placed inside of small, open foam cushion rings that were adhered to the ears beforehand. The mites were attracted to the ear warmth at the bottom of the well so they did not crawl out. One to 15 specimens were allowed to penetrate at separate mapped locations on each ear so that no interaction between individual specimens occurred. The mites did not irritate the rabbits so the mites were not disturbed or removed by the rabbits' grooming. The mites that burrowed into the skin were visible through the stratum corneum when viewed with a stereoscope. Each burrowed mite was examined every 20–24 hr and its activity noted. Periodically, after a molt was observed, some specimens were removed from the burrows, mounted, and examined using a compound microscope to verify the subsequent life stage. In addition, quiescent stages were mounted and examined with a compound microscope to identify their life stage and to determine what life stage was contained inside.

Egg hatching experiments were conducted *in vitro*. Mites were allowed to crawl from freshly removed mite-laden crusts onto a petri dish. Newly deposited eggs were removed from the dish, placed on black Metricel

filter paper on glass slides, and kept in a closed petri dish at 35 C at 100% RH. All eggs were less than 2 hr old; some were collected as they were deposited by the female. The eggs were examined microscopically every 1–2 hr during the day and evening until larvae emerged.

RESULTS

The mean incubation times (±SD) for freshly deposited eggs held at 100% RH and 35 C prior to hatching were >50.1 ± 2.45 but <52.97 ± 3.26 hr (n = 41) (Fig. 1). Twenty-four, 56, and 20% of eggs hatched during the incubation time intervals 44–50, 51–58, and 48–53 hr, respectively. Eighty-three percent of all eggs (n = 60) that were randomly selected within 2 hr of oviposition were observed to hatch. All gave rise to a hexapodal larva.

Mean elapsed time before burrowed larvae molted to the protonymphal stage was between 3.22 ± 1.52 and 4.20 ± 1.52 days (n = 40). Forty-eight percent of individual larvae molted to protonymphs in 3–5 days, 38% in less than 3 days, and 15% required longer than 5 days. Some of these protonymphs that emerged from larvae remained in the skin, continued to feed and burrow without interruption, and eventually molted to tritonymphs. The combined duration of both the larval and protonymphal stages for these specimens was between 6.5 ± 1.29 and 7.5 ± 1.29 days (n = 18). The duration of the protonymphal stage alone was between 2.89 ± 1.49 and 3.83 ± 1.58 days (Fig. 1).

Protonymphs that were removed from fresh crusts and allowed to burrow into rabbit skin molted to tritonymphs after 2.37 ± 0.70 to 3.37 ± 0.70 days (n = 178) from the time of infestation. Durations of protonymphal stages that were destined to become females and males were >2.40 ± 0.84 to <3.40 ± 0.84 and >2.33 ± 0.66 to <3.33 ± 0.66 days, respectively. Fifty-two percent of specimens molted in less than 3 days, whereas 48% required longer than 3 days before molting occurred.

Most specimens surfaced frequently during development, so it was not possible to continually follow all specimens through their entire life cycle. In one series of experiments, some specimens developed from protonymph to tritonymph to adult without ever leaving the original burrow. Duration of the combined development of protonymphal and tritonymphal stages of these specimens was between 5.67 ± 2.35 and 6.67 ± 2.35 (n = 9) with duration of the tritonymphal stage being between 2.00 ± 1.12 and 2.78 ± 1.39

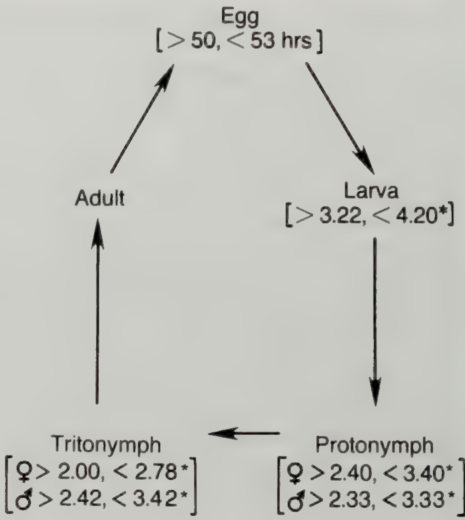

FIGURE 1. Life cycle of *Sarcoptes scabiei* var. *canis*; * = duration in days.

days and the protonymphal stage being between 2.89 ± 1.36 and 3.89 ± 1.36 days (Fig. 1). By comparison tritonymphs that were removed from fresh crusts and allowed to burrow into rabbit skin molted after 2.22 ± 1.01 to 3.42 ± 0.51 days (n = 90). Durations of tritonymphs destined to become females and males were >2.22 ± 1.01 to <3.22 ± 0.97 and >2.42 ± 0.51 to <3.42 ± 0.51 days, respectively. Fifty-five of these molted in less than 3 days, whereas 35 required longer than 3 days.

DISCUSSION

This study represents the first systematic *in vivo* investigation of the complete life cycle of *S. scabiei*. The results clearly indicate that the life cycle of both males and females of *S. scabiei* var. *canis* consists of 3 active developmental instars. Larvae emerge from eggs and give rise to protonymphs. The protonymphs give rise to tritonymphs from which either males or females emerge.

These *in vivo* results are in contrast to several previous reports. Although no data and methods are provided, Gordon and Lavoipierre (1962) and Van Neste et al. (1981) report that the life cycle of var. *hominis* consists of only 1 nymphal instar that gives rise to both males and females. Munro (1919) reported that male var. *hominis* emerge from a small first nymph (obtained on 2

occasions) and females emerge from a second larger nymph. Our observations of var. *canis* are consistent with the known development for Sarcoptidae and the Astigmata in which both protonymphs and tritonymphs occur in the development of both sexes (O'Connor, 1982). Therefore, var. *hominis* development is inconsistent with that of other Sarcoptidae or a life stage was overlooked. Because the male tritonymph is small and only slightly larger than the protonymph, this life stage may be confused with the protonymph upon examination with a stereoscope. Based on detailed examination of preserved specimens, Fain (1968), on the basis of differences in leg setae and solenidia, described both protonymphs and tritonymphs. Quiescent tritonymphs contained either a male or female. Our examination also revealed both males or females within quiescent tritonymphs and only tritonymphs within quiescent protonymphs. Our *in vivo* experiments revealed that small tritonymphs gave rise to males and large tritonymphs gave rise to females. It is likely that all strains of *S. scabiei* exhibit development similar to var. *canis* but this remains to be verified.

Development from egg to adult requires 10.06–13.16 days for the male and 9.93–13.03 days for the female (Fig. 1). Except for the eggs, the duration of all active developmental stages was determined *in vivo*. The duration of each life stage represented the time interval from the end of one molt (emergence) to the end of the next (emergence again). Therefore, it included a short quiescent period typical of mite development that precedes each molt. The duration of this quiescent period could not be determined *in vivo* because normally burrowed mites were very inactive and it was difficult to determine the onset of quiescence through the layer of stratum corneum above the burrowed mites. Eggs were incubated *in vitro* at temperature and humidity conditions similar to those of rabbit skin. Therefore, although details concerning development, activity in the borrow, and molting are lacking, the duration of each life stage as well as the entire life cycle and the identification of various developmental stages were clearly defined.

One of the problems encountered in tracing the development of *S. scabiei in vivo* was that all life stages frequently surfaced from the burrow and wandered on the skin surface. Some burrowed in new locations and others were lost and could not be tracked. For example, in one series of experiments, 26% of the monitored burrowed mites molted, whereas 9% died in the burrow and 65% wandered from the burrow and were lost ($n = 541$). In another series of observations, spanning across more than 1 instar, 9% died and 91% left their burrows, wandered, and were lost ($n = 374$).

Although many mites could not be followed beyond 1 complete life stage, a large number of specimens of each life stage were observed from beginning to end. In addition, the average duration of the larval, protonymphal, and tritonymphal instars that were randomly selected from fresh host crusts and placed on the new host were practically identical to the duration of similar instars that were followed from molt to molt *in vivo* following emergence from the preceding instar.

There are considerable differences of opinion reported in the literature regarding the duration and other details of the life cycle of *S. scabiei*. Munro (1919) and Van Neste et al. (1981) report the life cycle of var. *hominis* to be 7–10 days and 9–15 days, respectively. Bourgiugnon (1850 *in* Munro, 1919) and Furstenburg (1861 *in* Munro, 1919) give the normal period of egg hatching to be 10 days and 6–7 days, respectively, for var. *hominis*. By contrast, Megnin (1875 *in* Munro, 1919), Gerlack (1857 *in* Munro, 1919), and Munro (1919) report much shorter incubation times of 48, 64–76, and 67–103 hr, respectively. By comparison, in the current investigation, the incubation period for var. *canis* eggs at skin temperature and humidity conditions was between 50 and 53 hr, and it exhibited very little variation under constant conditions. It is not clear if the apparent differences between our results and those of older studies are attributed to strain differences or differences in experimental conditions.

It has been suggested that under some conditions var. *hominis* and var. *ovis* of sheep are viviparous or ovoviviparous (Munro, 1919; Nitzulescu, 1973). However, during the microscopical examination of numerous freshly killed gravid females, we have never observed females pregnant with larvae or eggs containing larvae. We, therefore, conclude that viviparity or ovoviviparity do not normally occur for *S. scabiei* var. *canis* and probably not for *S. scabiei* in general. The reported isolated cases of larvae observed inside of females represent instances in which a gravid female died but the egg inside her body continued to incubate, develop, and in some cases hatch. We have made similar observations in other mite species (Arlian and Woolley, 1970).

ACKNOWLEDGMENT

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BOOK REVIEW . . .

Ticks and Tick-Borne Diseases, E. D. Sutherst (ed.). ACIAR Proceedings, No. 17. Argyle Press, Brisbane. 1986. 159 p.

"Ticks and Tick-Borne Diseases," edited by E. D. Sutherst, contains the proceedings of an international workshop on the ecology of ticks and epidemiology of tick-borne diseases held at Nyanga, Zimbabwe in February 1986. The proceedings provide current information on the status of ticks and tick-borne diseases in Africa and Australia. Use of computer modelling to assess the impact of these parasites on cattle production is emphasized. The book is divided into five sections, the first of which summarizes the current status of the ecology, epidemiology, and control strategies of ticks in several African countries and Australia. The second section, "Tick Ecology," explores the use of computer modelling as a tool for understanding the complexity of tick survival and control within a given environment. The specific requirements of each tick developmental phase (free-living, host-finding, and parasitic) are considered separately in these models. Epidemiology of tick-borne diseases, primarily theileriosis, is reviewed in the third section. Recommendations of needs for diagnostic tests and computer models are summarized in this section. The fourth section is devoted to losses in production resulting from ticks and tick-borne diseases. Recommendations include development of strategies to evaluate and document production losses of livestock. The final section on Management explores current management strategies. Recommendations emphasize incorporation of ecological and epidemiological models as management tools. The proceedings contain current information and provide recommendations for future research and management formulated by top experts in the field. The tremendous impact and importance of ticks and tick-borne diseases on development of Africa cannot be overstated. These proceedings begin to fill a void in information on the impact of ticks in many African countries. It also provides a reference for understanding the complex epidemiology of ticks and is highly recommended for those interested in computer modelling of tick biology and tick-borne diseases.

Katherine M. Kocan, Department of Veterinary Pathology, Oklahoma State University, Stillwater, Oklahoma 74078.

J. Parasit., 74(3), 1988, p. 431

CORRIGENDA . . .

Due to an inadvertent error, a Research Note entitled "Late Fall Transmission of *Nematodirus battus* (Nematoda: Trichostrongyloidea) in Western Oregon" by Lora G. Rickard, Eric P. Hoberg, Gary L. Zimmerman, and Janell K. Erno was published twice. The correct citation for this Research Note is: Journal of Parasitology **73**(1): 244.

In the Subject Index for Volume 73 under *Echinostoma revolutum,* reference to an SEM study of the tegumentary papillae on the cercariae was accidently omitted.

J. Parasit., 74(3), 1988, pp. 432–439

ULTRASTRUCTURE OF THE ENCAPSULATION OF *PLASMODIUM CYNOMOLGI* (B STRAIN) ON THE MIDGUT OF A REFRACTORY STRAIN OF *ANOPHELES GAMBIAE*

Susan M. Paskewitz*†, Mark R. Brown, Arden O. Lea, and Frank H. Collins*
Department of Entomology, University of Georgia, Athens, Georgia 30602

ABSTRACT: Using transmission electron microscopy, we investigated the encapsulation of the simian malaria parasite, *Plasmodium cynomolgi,* in a refractory strain of the mosquito, *Anopheles gambiae.* After the ookinete penetrates the mosquito midgut epithelium and lodges between the basal membrane and the basal lamina, an electron-dense, melanin-like substance begins to coalesce around the parasite. Completely encapsulated parasites were found as early as 16 hr after the blood meal. Granules of the melanin-like substance often appeared to condense onto the parasite from the fluid in the extracellular spaces of the basal membrane labyrinth. Melanin granules also appeared to condense from the hemolymph onto the basal lamina underlying the parasite. In addition, groups of tubules, vesicles, and membranous whorls often were found in midgut cells that were located next to or were enclosing parasites. These structures were unusually electron-dense, and may have been associated with melanization. Hemocytes rarely were observed near completed capsules and neither hemocytes nor their remnants were components of the capsules. During later stages of encapsulation, parasites appeared abnormal and often were infiltrated with melanin. Although late-stage capsules were usually located basally, completed capsules enclosed by membranes were occasionally observed near the apical border of the midgut. Other capsules, associated with cellular debris, were found in the lumen of the midgut from 1 to 6 days after the blood meal.

Recently, a line of *Anopheles gambiae* refractory to several *Plasmodium* parasites, including *P. cynomolgi,* was selected from the parent strain that is susceptible to these parasites (Collins et al., 1986). Within 24 hr after a refractory mosquito takes an infective blood meal, amber to black-colored bodies, 5–10 μm wide, dot the mosquito midgut. No sporozoite development occurs in these mosquitoes. Somewhat similar bodies, known as "Ross's black spores," are sometimes found when malaria parasites develop in unusual mosquito hosts or when a normal host is exposed to abnormal temperature regimes (Ross, 1898; Daniels, 1899; Mayne, 1929; Garnham, 1966; Shute and Maryon, 1966).

Other than the degenerate oocyst enclosed in a thin, electron-dense capsule illustrated in Sinden and Garnham (1973), no ultrastructural studies of this defensive response to malaria parasites exist. Several such studies have been made of mosquito encapsulation of filarioid parasites in the hemocoel (Bradley et al., 1984; Chen and Laurence, 1985; Forton et al., 1985; Christensen and Forton, 1986). These studies indicate that hemolymph and hemocytes are involved in encapsulation responses. Other studies have shown that capsules may form around filarial worms while in muscle or Malpighian tubule cells (Lehane and Laurence, 1977; Christensen, 1981).

We undertook the present study to investigate how mosquito tissues, especially midgut and hemolymph, are involved in capsule formation on the midgut of *An. gambiae.*

MATERIALS AND METHODS

The refractory strain and a susceptible strain, which were selected from the same parental line of *An. gambiae,* were reared as described by Vanderberg and Gwadz (1980). Mosquitoes were 3–7 days old when fed on rhesus monkeys (*Macaca mulatta*) infected with *P. cynomolgi* B (for a description of the B strain see Coatney et al., 1971).

Mosquito midguts were dissected in fixative (2% glutaraldehyde in 0.05 M Na-phosphate or cacodylate buffer containing 2% sucrose, pH 7.6) at 4 C. Infected guts were fixed at 12, 16, 20, and 24 hr and at 6 and 8 days after the blood meal. After an 8–12-hr fixation at 4 C (to fix the blood meal), midguts were washed in 0.05 M Na-phosphate or cacodylate buffer containing 7% sucrose. Postfixation was in 1% OsO_4 in 0.05 M Na-phosphate or cacodylate buffer containing 2% sucrose, pH 7.6. Midguts were dehydrated in a series of increasingly concentrated ethyl alcohol dilutions, infiltrated with a mixture of propylene oxide and Epon–Araldite, and embedded in Epon–Araldite. Thick sections (0.5–1 μm) were cut with glass knives and stained with toluidine blue. When capsules were observed with light microscopy, thin sections were cut on a Sorvall MT-2B ultramicrotome. Grids were stained with lead citrate followed by uranyl acetate and examined with

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* Malaria Branch, Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333.

† Address correspondence and reprint requests to S.M.P. at CDC.

a Philips 400 electron microscope. At least 3 midguts were sectioned for each time period, and a total of 68 encapsulated parasites were observed.

With the understanding that we did not make the chemical tests necessary to prove that the electron-dense capsule substance is melanin, we call this substance "melanin" throughout the text. This seems reasonable because other studies of encapsulation by nematocerous Diptera have shown that similar substances have a melanin component (Poinar and Leutenegger, 1971; Vey and Gotz, 1975).

RESULTS

The blood meal, containing infective stages of *P. cynomolgi,* enters the abdominal midgut after ingestion. Most encapsulated parasites were found on the abdominal midgut, although a few were located on the posterior one-third of the thoracic midgut. The abdominal midgut is a monolayer consisting of digestive, endocrine, and regenerative cell types similar to those found in other mosquitoes (Hecker, 1977; Houk, 1977). Digestive cells are the most common and were the only cells associated with malaria parasites.

Ookinetes in refractory and susceptible mosquitoes were morphologically similar to those observed in previous studies of this and other malaria species (Garnham et al., 1962, 1969). Characteristic ookinete organelles, such as the crystalline body, micronemes, heme pigment bodies, and the subpellicular microtubules, were always observed (Fig. 1). Ookinetes lodge between the basal membrane labyrinth and the basal lamina after crossing the midgut. Ookinetes in susceptible mosquitoes continued to develop normally and produced infective sporozoites around 12 days after the blood meal.

Early stages of encapsulation

Refractory mosquitoes began to respond to and encapsulate ookinetes after the parasites penetrated the midgut epithelium. The earliest capsules ("capsule" refers to a parasite that is completely surrounded by melanin) were visible by 16 hr after the blood meal. The first indication of melanization was a thin coat of electron-dense particles around an ookinete (Figs. 2, 3); the ookinete often was located in an extracellular matrix between the basal membrane of a midgut cell and the basal lamina (Fig. 3). When small vesicular bodies were detected in the extracellular space with the parasite, they were also melanized (Fig. 3). Most of the parasites were completely encapsulated within 24 hr after feeding (Fig. 4).

Granules of capsule substance from the hemolymph often appeared to condense onto the basal lamina near the parasite (Figs. 2, 5). Capsules usually were thickest on the hemocoel side, with a "cap" of melanin over the basal lamina (Fig. 5). The melanin capsule around the oocyst illustrated in Sinden and Garnham (1973) is also thickest on the hemocoel side.

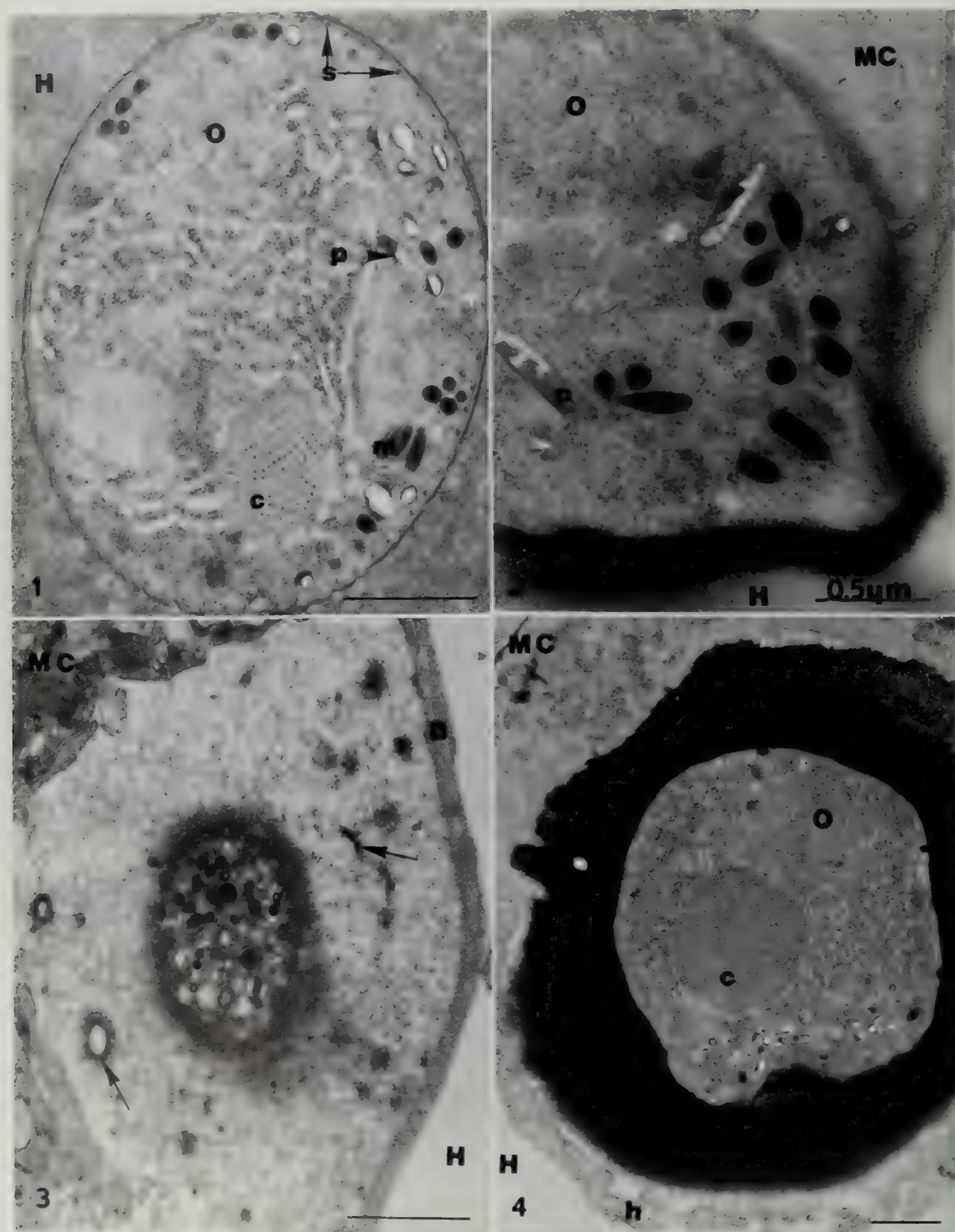
Single hemocytes (or remnants) were seen near only 5 of the 68 capsules observed and were always in association with later, rather than earlier, stages of capsule formation (Fig. 6, see also Fig. 4). On 2 of the 5 occasions, hemocytes had lysed in the vicinity of a completed capsule.

Small tubules and vesicles occasionally were observed in midgut cells located near parasites (Figs. 7, 8). Some of these structures were lamellate (Fig. 7). Similar structures were found near ookinetes that were still within midgut cells (Figs. 8a, b). Many of the structures contained electron-dense material, indicating a possible role in the formation of melanin (Figs. 7, 8). Some of the vesicles in Figures 7 and 8 resemble parts of the nearby basal membrane labyrinth, suggesting that the tubules and vesicles may actually be a part of these infoldings of the basal membrane, rather than inside the midgut cells. Lamellate tubules and vesicles containing electron-dense material were not observed in uninfected cells of refractory midguts, nor were they ever seen in the susceptible strain.

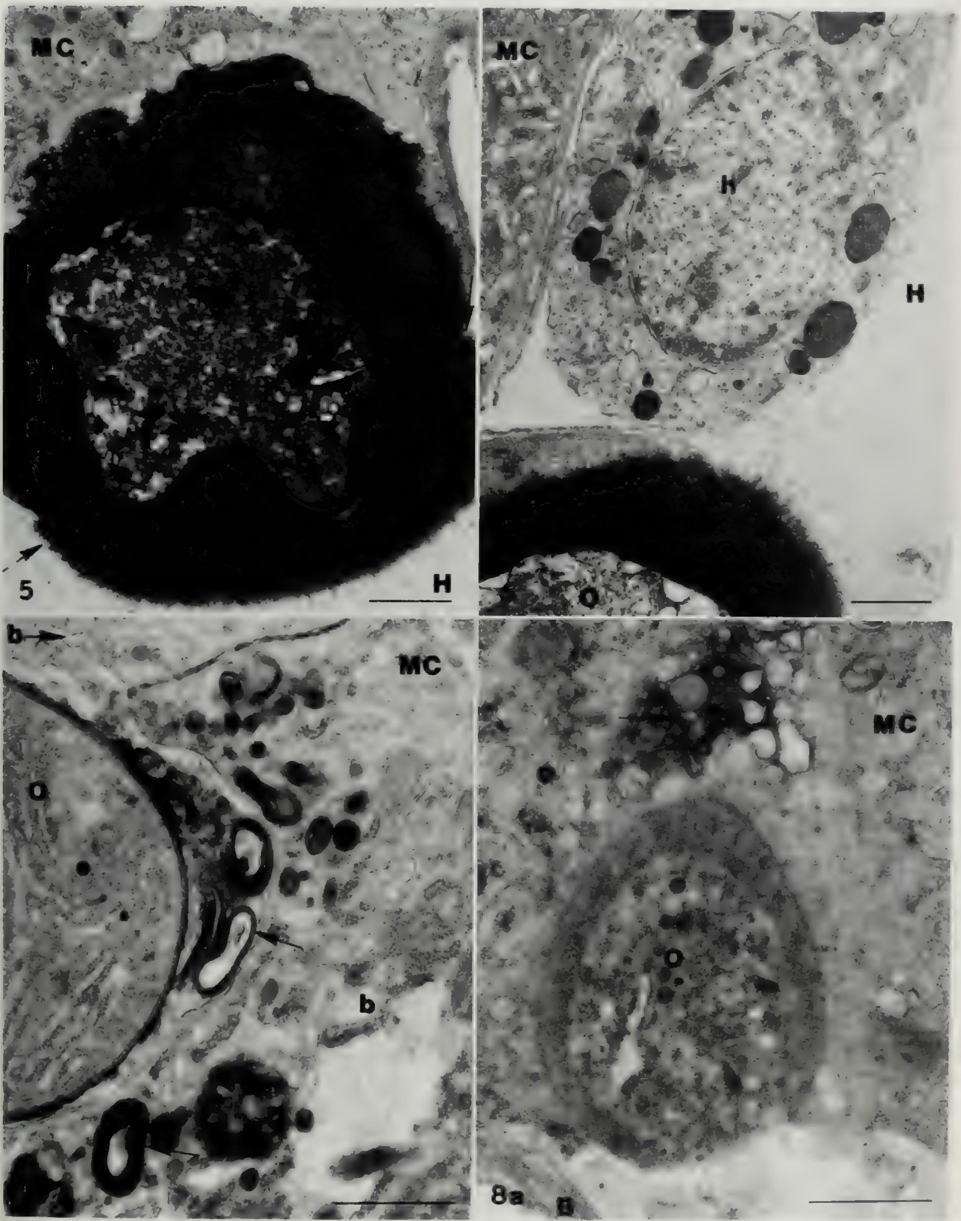
Late stages of encapsulation

Completed capsules were 5–10 μm thick. Parasite structure and organelles usually appeared normal until after the capsule substance enclosed them entirely. By 24 hr after the blood meal many parasites were infiltrated with melanin, and by 8 days after the blood meal parasite structure was usually completely obscured by melanin (Fig. 9). Ookinetes sometimes ruptured during encapsulation, providing an entry for melanin. Other abnormalities, including formation of large membranous whorls inside the ookinete and vacuolization of the parasite cytoplasm, also were seen.

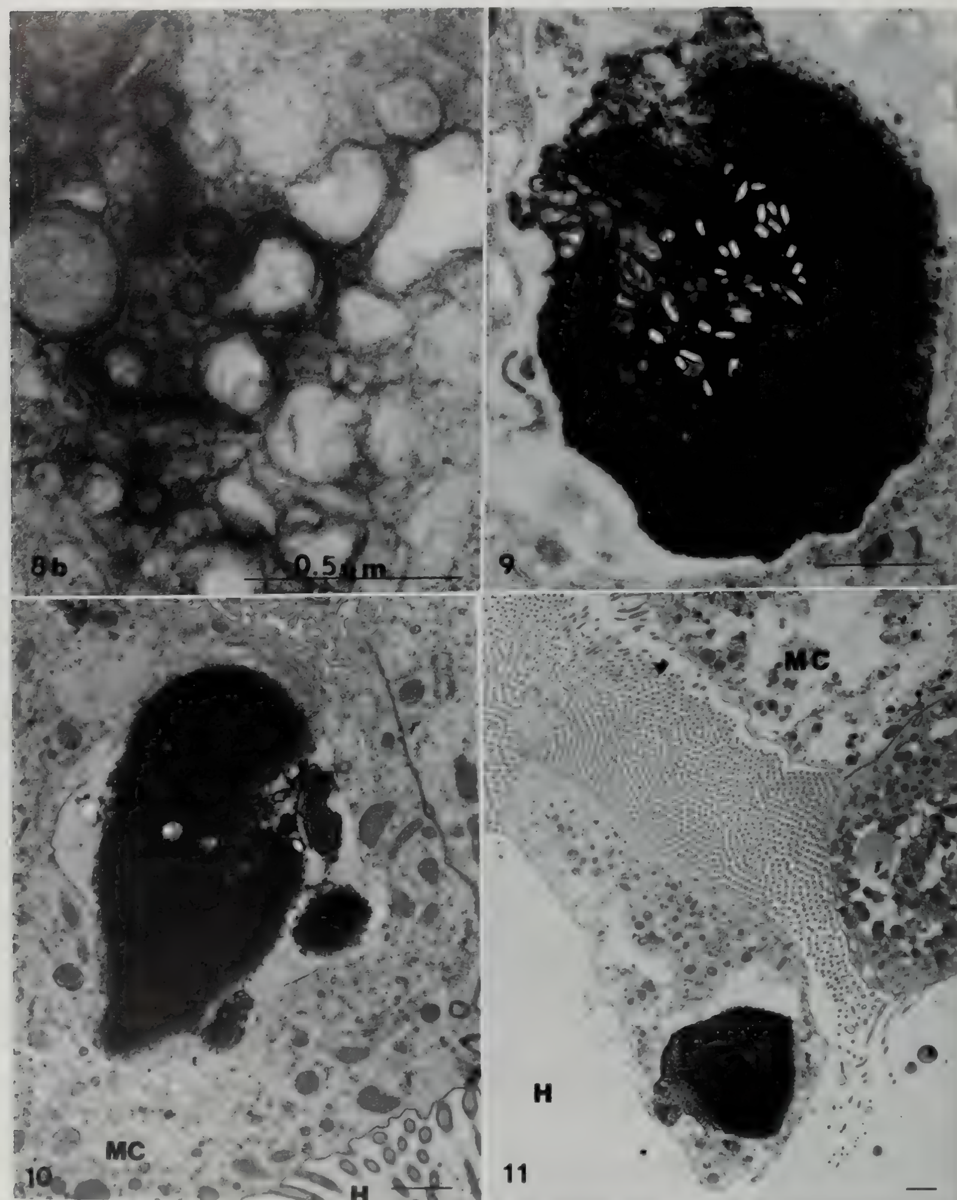
Although most capsules were observed in the basal area of the midgut epithelium, 6 encapsulated parasites were found enclosed by membranes in the central or apical portion of the midgut epithelium (Fig. 10). One of these parasites was found at 24 hr after the blood meal; the others were observed at 6 days after blood-feeding. Three encapsulated parasites were found



FIGURES 1–4. Formation of melanotic capsules around *P. cynomolgi* ookinetes in refractory *An. gambiae*. Bar = 1 μm. **1.** Normally developing ookinete at 20 hr after a blood meal. **2.** Melanin deposition on the side of the ookinete near the hemocoel (arrow). **3.** Early stages of melanin deposition. Note the dense matrix around the vesicles and membranes in the space with the ookinete (arrows). **4.** Fully encapsulated ookinete at 24 hr after an infective blood meal. List of abbreviations: B, basal lamina; b, basal membrane labyrinth; c, crystalline body; H, hemocoel; h, hemocyte; m, microneme; MC, midgut cell; O, ookinete; p, heme pigment body; s, subpellicular microtubules.



FIGURES 5–8a. Formation of melanotic capsules around *P. cynomolgi* ookinetes in refractory *An. gambiae*. Bar = 1 μm. **5.** Note the melanin "cap" covering the capsule and basal lamina (arrows). Melanin deposition is heaviest on the hemocoel side. **6.** Hemocyte adjacent to encapsulated parasite. **7.** Tubular system in midgut cell associated with ookinete. Note darkened whorls in cytoplasm (arrow). **8a.** Vesicular system associated with unencapsulated ookinete. See Figures 1–4 for list of abbreviations.



FIGURES 8b–11. Formation of melanotic capsules around *P. cynomolgi* ookinetes in refractory *An. gambiae.* Bar = 1 μm unless otherwise noted. **8b.** Higher magnification of vesicular system. Note darkening and lamellar structures. **9.** Late-stage parasite infiltrated with melanin. **10.** Capsule in apical portion of midgut epithelium, probably between 2 cells, being expelled to lumen. **11.** Capsule associated with (inside of?) degenerating cell in midgut lumen. See Figures 1–4 for list of abbreviations.

within the midgut lumen. One of these was associated with a disintegrating cell that had been released into the lumen (Fig. 11) and was found 6 days after the blood meal. The other 2 were associated with cellular debris and were found at 1 and 6 days after the blood meal. Neither ectopic nor intracellular development of oocysts was observed in the susceptible strain.

DISCUSSION

Ookinetes of *P. cynomolgi* are melanotically encapsulated between the midgut basal membranes and the basal lamina in a refractory strain of *An. gambiae*. The origin of the melanin component of the capsules was not clear. Hemocytes, which contribute to capsule formation in some adult mosquito–filarid systems (Forton et al., 1985), were rarely seen during the present study. Neither intact hemocytes nor their remnants were physically part of the capsules, even that part (the "cap") which formed on the hemocoel side of the basal lamina. However, it is possible that hemocytes, lying near ookinetes or capsules but not attached to the midgut, were lost during preparation for electron microscopy. Hemocytes might release substrates or enzymes for melanogenesis, or activators of these enzymes, near the ookinetes. Any material thus released would have to pass through the basal lamina from the hemocoel to the parasite. Other studies have shown that hemocytes play an important role in initiating formation of a protein–polyphenol complex leading to melanization in some adult mosquito–filarid systems (Forton et al., 1985; Christensen and Forton, 1986). These parasites, however, are not contained by the basal lamina but are free in the hemocoel.

Alternatively, the hemocytes observed in *An. gambiae* may be responding to capsules that have already formed from components originating in the tissue or the hemolymph. Chen and Laurence (1985) have reported that, in encapsulation of filarial worms in *Anopheles quadrimaculatus*, a layer of melanin is laid down 2–3 hr before hemocytes adhere to the capsule.

If the latter is true, then the necessary enzymes or substrates may be present in the hemolymph or extracellular spaces of the basal membrane labyrinth or in the midgut cells themselves. In the larvae of other nematocerous Diptera (*Chironomus, Culex*), a form of humoral encapsulation of fungi and nematodes has been reported, where capsular substances arise from the hemolymph without hemocyte involvement (Gotz and Vey, 1974; Vey and Gotz, 1975). Several studies have reported that adult mosquito tissues are capable of melanization responses to filarial worms. These tissues include muscle fiber cells (Lehane and Laurence, 1977), fat body (Bartlett, 1984), and Malpighian tubule cells (Christensen, 1981). The electron-dense material seen in lamellate tubules and vesicles of the midgut near parasites may indicate midgut cell participation in the reaction. Parts of the vesicular systems resemble the basal labyrinth but the lamellate structure of some of these vesicles does not, so the relationship of these vesicles to midgut cells is not clear.

During later stages of encapsulation, capsules are found in the lumen of the midgut epithelium. Capsules may get to the lumen through openings created during the turnover of old cells after a blood meal. These degenerate cells were frequently observed in all stages of being released to the lumen. Shute and Maryon (1966) noticed a decrease, with time, in the number of "Ross's black spores" on mosquito midguts, which could have been due to a similar "crowding out." It seems most likely that the membrane seen around those capsules that were located apically within the epithelium originates from midgut cells on either side of the capsules. However, it may be that these parasites were actually encapsulated initially within midgut cells. Some encapsulated parasites were seen associated with (inside of?) disintegrating cells in the lumen. Beaudoin et al. (1974) discovered oocysts developing normally within midgut cells. These authors also observed several normal oocysts within the midgut lumen and found that feces from some mosquitoes contained infective sporozoites. And, as mentioned above, there are other reports of melanization of filarial worms within mosquito cells. No ectopic or intracellular development of oocysts or ookinetes was identified in the susceptible strain.

The process of encapsulation that leads to the formation of "Ross's black spores" may be similar to what we have described. "Ross's black spores" are described as yellow to brown, round, or, more commonly, banana-shaped capsules occurring singly or in clusters on the midgut (Ross, 1898; Daniels, 1899; Mayne, 1929; Garnham, 1966; Shute and Maryon, 1966). The "black spores" have not been reported until 3 days after the blood meal, although the literature does not indicate whether blood-filled midguts had been

examined earlier. Because the sporozoites, developing inside oocysts, are often outlined with melanin, resulting in the typical "banana" spores (Garnham, 1966), encapsulation probably does not begin before at least 3 days. Formation of "Ross's black spores" is generally considered to occur around a dead or dying parasite that had developed normally for several days. In the refractory *An. gambiae* system, encapsulation begins almost immediately, apparently in response to normal ookinetes. The few parasites that bypass the encapsulation response develop normally (Collins et al., 1986). Thus, we feel that although the mechanisms of melanin formation for "Ross's black spores" may be similar, the response by the refractory *An. gambiae* immune system to the parasite is an active response to an invader in this system, rather than a passive encapsulation of a dead object.

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CHARACTERIZATION OF THE ACCESSORY LAYER OF THE CUTICLE OF MUSCLE LARVAE OF *TRICHINELLA SPIRALIS*

K. A. Wright and H. Hong
Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

ABSTRACT: The accessory layer of the cuticle of infective larvae of *Trichinella spiralis* has been studied with electron microscopy using cytochemical techniques and chemical extractions. The accessory layer lacks negative charges and carbohydrates demonstrable *in vivo*. Staining with ruthenium red and tannic acid is interpreted as being consistent with their reactions with phospholipids. Freeze fractures demonstrate an external layer of granules that can be partially released by means of detergents (CTAB and SDS). The granules are considered to be proteins. Their removal makes the worms acid sensitive and prevents them from infecting mice. Extraction of whole worms with ethanol, acetone and methanol (via reaction with 2,2-DMP), or chloroform and methanol destroys an internal layer of filaments. Thin-layer chromatography of chloroform/methanol extracts showed principally ethanolamine phospholipids from the surface of the worms. A model is presented for the molecular organization of the accessory layer. Ethanolamine phospholipids are suggested to occur as tubular micelles. Proteins may attach to these by lipophilic moieties and perhaps by a cryptic sugar group (demonstrated by others) that may penetrate into the hydrophilic core of the lipid micelles.

The surface layers of the cuticle of the infective (L_1) larvae of *Trichinella spiralis* are unusually complex (Lee et al., 1984). In addition to the cuticle's epicuticle, there is an external layer of material that appears after 9–10 days of the worm's development within the muscle nurse cell and remains until the first moult within the intestinal epithelium (Despommier, 1983). Subsequent stages of development lack this extra external layer (Lee et al., 1986). It has recently been suggested that this layer be recognized as an accessory component of the worm's cuticle (Wright, 1987). Preservation of the accessory layer is minimal by means of routine fixation regimes using glutaraldehyde and osmium. It was first resolved by electron microscopy as 2 dense lines in thin sections of muscle tissue preserved in a osmium–dichromate fixative (Purkerson and Despommier, 1974). More recently, freeze-fracture preparations revealed an outer component of globules that appeared to be enclosed in a smooth-surfaced matrix, and an inner component comprised of long closely packed filaments or tubules (Lee et al., 1984). Although it was then speculated that part, or all, of the accessory layer could be lipid, it is clearly not a cell membrane because of its distinctive fracturing characteristics. This study has been extended to include cytochemistry and chemical extraction experiments to further characterize the accessory layer.

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MATERIALS AND METHODS

Source of nematodes

Larvae were recovered from muscles of CD-1 mice by digestion with 1.5% pepsin in 1% HCl for 1–2 hr. They were washed in Hanks' balanced salt solution (HBSS) as in previous studies (Lee et al., 1984).

Electron microscopy

Following various treatments, worms were fixed at room temperature in 1.6% glutaraldehyde in cacodylate buffer or 1.7% glutaraldehyde in PIPES buffer with an osmolality adjusted to 300 mOsm or less. After several hours of fixation at room temperature, worms were stored at 5 C in fixative for variable times. When appropriate, the worms were postfixed in 1% buffered osmium tetroxide at room temperature for 1–2 hr. Those prepared for thin sections were processed by standard methods of dehydration with ethanol and embedding in Spurr's resin. For freeze fracture, the worms were rinsed free of the glutaraldehyde fixative with buffer and gradually infiltrated with a 30% solution of glycerol in distilled water. However, worms that were to be extensively etched after fracturing were kept in distilled water. Following freezing in a Freon 22 slush and storage in liquid nitrogen, fractures were made by standard methods in a Balzer's BAF 301 or 400. Etching was performed at -98 C under 10^{-6} Torr for 5 min.

Although most tissues were fixed in glutaraldehyde buffered with cacodylate or PIPES, those fixed with 1% tannic acid were buffered with 0.1 M Sorensen's phosphate buffer. Worms were fixed at room temperature for about 3 hr, then stored overnight at 5 C. After repeated rinsing with buffer, they were osmicated in 1% osmium tetroxide in phosphate buffer for 1.25 hr. Ruthenium red (0.8%) was also included in cacodylate-buffered glutaraldehyde, and worms were fixed for the same times as those above. Ruthenium red (0.8%) was included in the osmication solution (1.25 hr). Worms to be stained by the PA-TSC-silver proteinate technique (Théiry, 1967) were fixed in cacodylate-buffered glutaraldehyde, but were not osmicated. A pellet of red blood cells freshly collected from the mouse host was

prepared similarly as a positive control for the PA-TSC-silver proteinate method. Control sections of worms were stained without oxidation by periodic acid, or were reacted with a 2% solution of dimedone in 1% acetic acid to blockade aldehyde groups formed by periodic acid oxidation. Worms to be stained with colloidal iron were fixed in 2% glutaraldehyde in cacodylate buffer for 2 hr at room temperature followed by 17 hr in fixative at 5 C. After rinsing in 12% acetic acid, they were reacted with positively charged iron colloid (Gasic et al., 1968) for 1.5 hr at room temperature, washed in buffer (pH 7.2), and osmicated in 1% osmium tetroxide for 1 hr. A pellet of freshly collected mouse red blood cells was treated similarly as a control.

Two collections of worms were prepared for freeze fracture by initial fixation for 15 min in 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer followed by fixation for 3 or more hours in glutaraldehyde/phosphate containing 1% dimethylsulphoxide and 0.04% filipin (Berdan and Shivers, 1985).

Worms were also dehydrated with 2,2-dimethoxypropane (2,2-DMP) following fixation with glutaraldehyde/cacodylate. Some were osmicated prior to this dehydration. For these treatments, 2,2-DMP was acidified by addition of 1 N HCl to 2,2-DMP (1:100 v/v) immediately prior to adding the acidified 2,2-DMP to worms in a minimum of buffer (Beckmann and Dierichs, 1982). The worms were freeze fractured or embedded in Spurr's resin for sectioning.

Extraction and digestion of surface materials

The effects of the detergents cetyl trimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) were determined initially as solutions in Eagles MEM (Pritchard et al., 1985), but mainly as solutions in HBSS. The worms were reacted with the detergents at 37 C on a rotator mixing device.

Larvae were also incubated at 37 C in 0.25% Bacto-trypsin (Difco) reconstituted with HBSS.

Lipids were extracted by means of a chloroform/methanol (2:1 v/v) solvent. Freshly cleaned living worms (5,000 per ml HBSS) were loaded into a 2-ml glass syringe along with 1 ml of air and flushed onto a 1.3-cm glass microfiber filter (Whatman 934-AH) in a Swinnex filter holder. The 1 ml of air was put through the filter to flush the HBSS from the holder. The syringe was quickly replaced with a 5-ml glass syringe containing 2 ml of the chloroform/methanol mixture and about 1 ml of air. This was flushed through the filter rapidly (<1 sec) or over a time of about 5 sec. Such rinses were pooled for processing by thin-layer chromatography. If these rinsed worms were to be freeze fractured, they were quickly fixed on the filter by passing buffered glutaraldehyde through the filter. The filter holder was then opened and worms were washed from the filter for further fixation. If the rinsed worms were to be extracted for total lipids, the filters with worms were placed in chloroform/methanol in which they were subsequently homogenized in a glass tissue grinder, according to the method prescribed by Folch et al. (1957).

Thin-layer chromatography

Chromatographic separation of lipids from rinses or extracts of worms was conducted as recommended by Fried and Sherma (1986). The chloroform/methanol extracts were mixed with a small volume of 0.58% NaCl to remove polar impurities, allowed to settle into a biphasic mixture, and the top hydrophobic layer was discarded. The remaining bottom lipid-containing layer was evaporated with dry nitrogen, resuspended in petroleum ether, and spotted onto silica gel-H plates (Supelco Redicoat 5-8144) prewashed by running in chloroform/methanol (1:1 v/v).

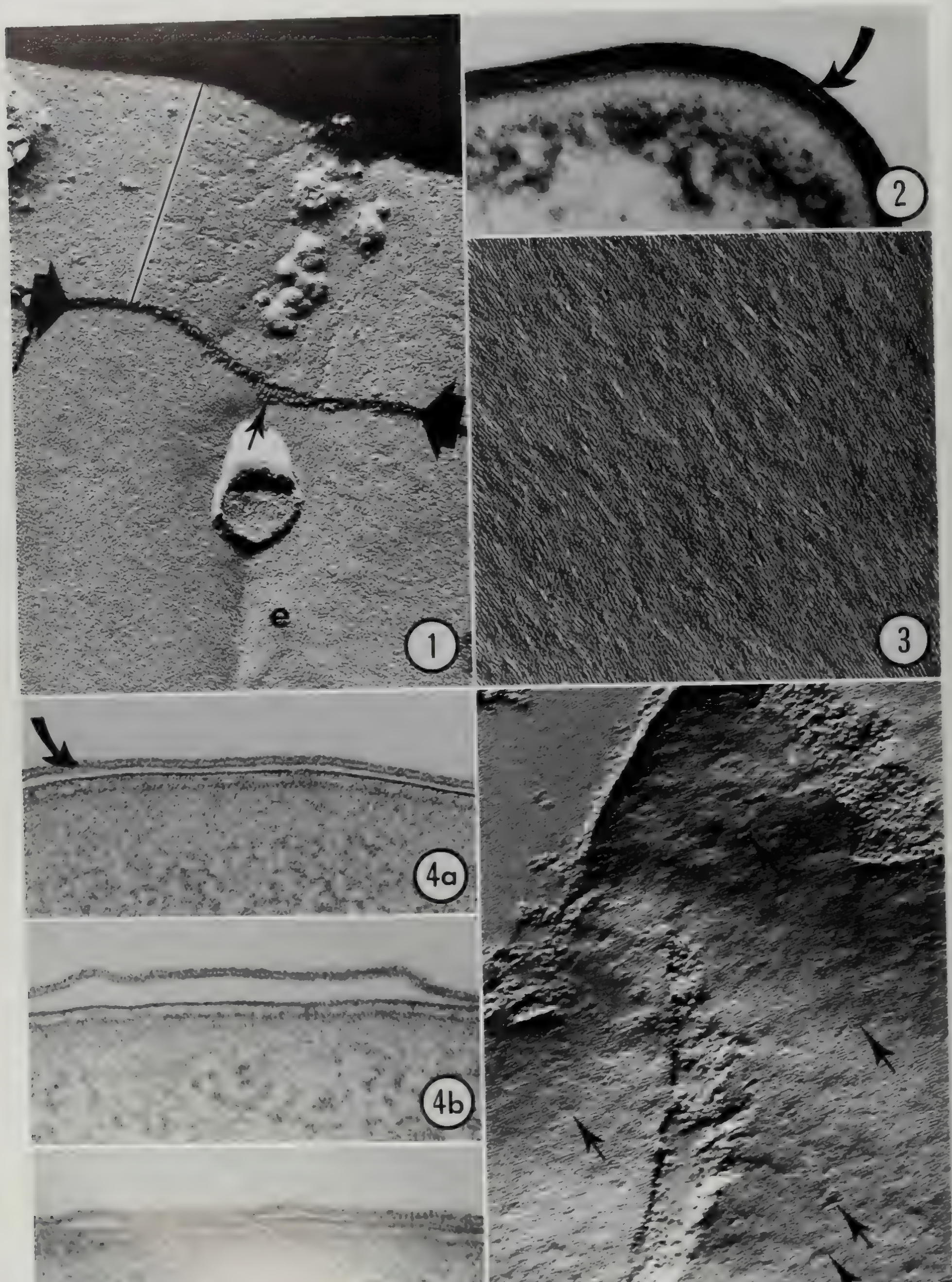
Plates were run in 2 dimensions. In the first dimension, the solvent system of petroleum ether : diethyl ether : acetic acid (90:9:1) separated neutral lipids from phospholipids. The solvent front was run one-third the height of the plate. The plate was removed from the developing tank, allowed to dry, and the control standards, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and phosphatidylserine (Sigma), were applied. In the second dimension, the solvent system of chloroform : methanol : acetic acid : water (50:30:8:4) separated the phospholipids that had remained at the origin. The above standards were chosen as they are the phospholipids identified by Castro and Fairbairn (1969) in *Trichinella*. A few controls with cholesterol (Sigma) were run in the first solvent. Plates were initially developed in iodine vapours, and subsequently sprayed with 5 M sulphuric acid in absolute ethanol (1:1 v/v) and charred in a 100 C oven.

RESULTS

Ultrastructural characterization

As noted previously (Lee et al., 1984), the accessory material is not always uniformly preserved in glutaraldehyde/osmium fixation when tissues are dehydrated for embedding in epoxy resins. Most frequently it appears in sections as a uniformly dense line (about 15 nm thick); sometimes it was resolved into a trilaminate pattern of 2 dense lines. Freeze fractures of glutaraldehyde-fixed worms consistently revealed the cleavage planes identified earlier, demonstrating an inner region of filaments with overlying globules. When fractures were etched for about 5 min prior to making the replica, the real surface of the accessory layer was shown to be the globules themselves (Fig. 1). In addition to the uniformly fine granularity of the etch-exposed surface, there was a range of patterns suggestive of either large globules or blisters and crater-like forms in this surface.

Inclusion of ruthenium red in the glutaraldehyde and osmium solutions has given a densely and uniformly stained accessory layer about 15 nm thick (Fig. 2). Freeze fractures of worms fixed by the glutaraldehyde ruthenium procedure gave fracture patterns of the accessory layer, cuticle, and internal tissues, very similar to those described previously. When the accessory material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was viewed from the inner surface, the filaments appeared very continuous (Fig. 3), without the fine gaps, nor the larger irregular areas, seen in worms fixed in glutaraldehyde only (Lee et al., 1984). In some areas, a pattern of granules or globules, rather than filaments was seen but it was less extensive than in worms kept longer at 37 C in salt solutions and fixed only in glutaraldehyde (Lee et al., 1984).

In worms fixed with glutaraldehyde and tannic acid, the accessory layer could be resolved as a trilaminar pattern about 15 nm thick (Fig. 4a). It was often closely applied to the surface of the cuticle, but in some areas it was elevated from the cuticle and had angular bends in it, suggesting that it was rigid and less flexible (Fig. 4b). In some places, slightly oblique alignment of the accessory layer in the section revealed a cross-banded pattern in it (Fig. 4c). Freeze fractures of glutaraldehyde/tannic acid-fixed tissue showed several distinctive features (Fig. 5). Most prominently, fractures showed irregular clusters of globules associated with the outer surface of the accessory layer. In the same material, filaments are quite well resolved on the inner surface, but there are numerous small indented areas (pocs) where filaments are not clearly resolved. In a few animals the filament pattern is faint, or replaced by a finely textured surface.

The cuticle of worms treated with negatively charged iron colloid and prepared as thin sections did not uniformly bind the iron. However, some strands of material, which may have been the accessory layer peeled off the surface, did have some iron granules attached. Mouse red blood cells were uniformly stained on their surfaces with the iron colloid.

Sections of glutaraldehyde-fixed worms stained with the PA-TSC-Ag proteinate method gave heavy staining of glycogen in epidermal, muscle, and intestinal tissues. However, there was no stain in the surface layers of the cuticle. Control sections (i.e., those not oxidized with periodic acid, or those treated with the aldehyde blockading reagent, dimedone) indicated that the technique was operating appropriately to detect periodate-formed aldehydes commonly demonstrable on carbohydrates. Mouse red blood cells showed a thin line of PA-TSC-Ag proteinate stain along their membrane surface.

The drug filipin complexes with and aggregates cholesterol in cell membranes, giving irregular bumps in freeze-fracture replicas. However, worms fixed with filipin showed no modifications in freeze-fracture patterns of the accessory layer, although the same sample of drug had been found to be very effective in other tissues (Berdan and Shivers, 1985).

Worms rapidly dehydrated by means of the reagent 2,2-DMP gave sections that illustrated cytoplasm with considerable density, but cell and organelle membranes as white lines (Fig. 6). This was presumably due to extraction of lipids from the membranes by the acetone and methanol formed *in situ* by reaction of 2,2-DMP with water in the worms' tissues (Beckmann and Dierichs, 1982). Worms fixed in glutaraldehyde and osmium prior to exposure to 2,2-DMP revealed the accessory layer of the cuticle as a continuous dense line (Fig. 6). However, in those worms fixed in glutaraldehyde, then reacted with 2,2-DMP, rehydrated, and osmicated later, the accessory layer was detected only as a fuzzy region above the epicuticle (Fig. 7a, b). Similar worms freeze fractured after glutaraldehyde fixation, and 2,2-DMP treatment (no osmium) showed only impressions of the accessory material (Fig. 8)—no filaments could be detected.

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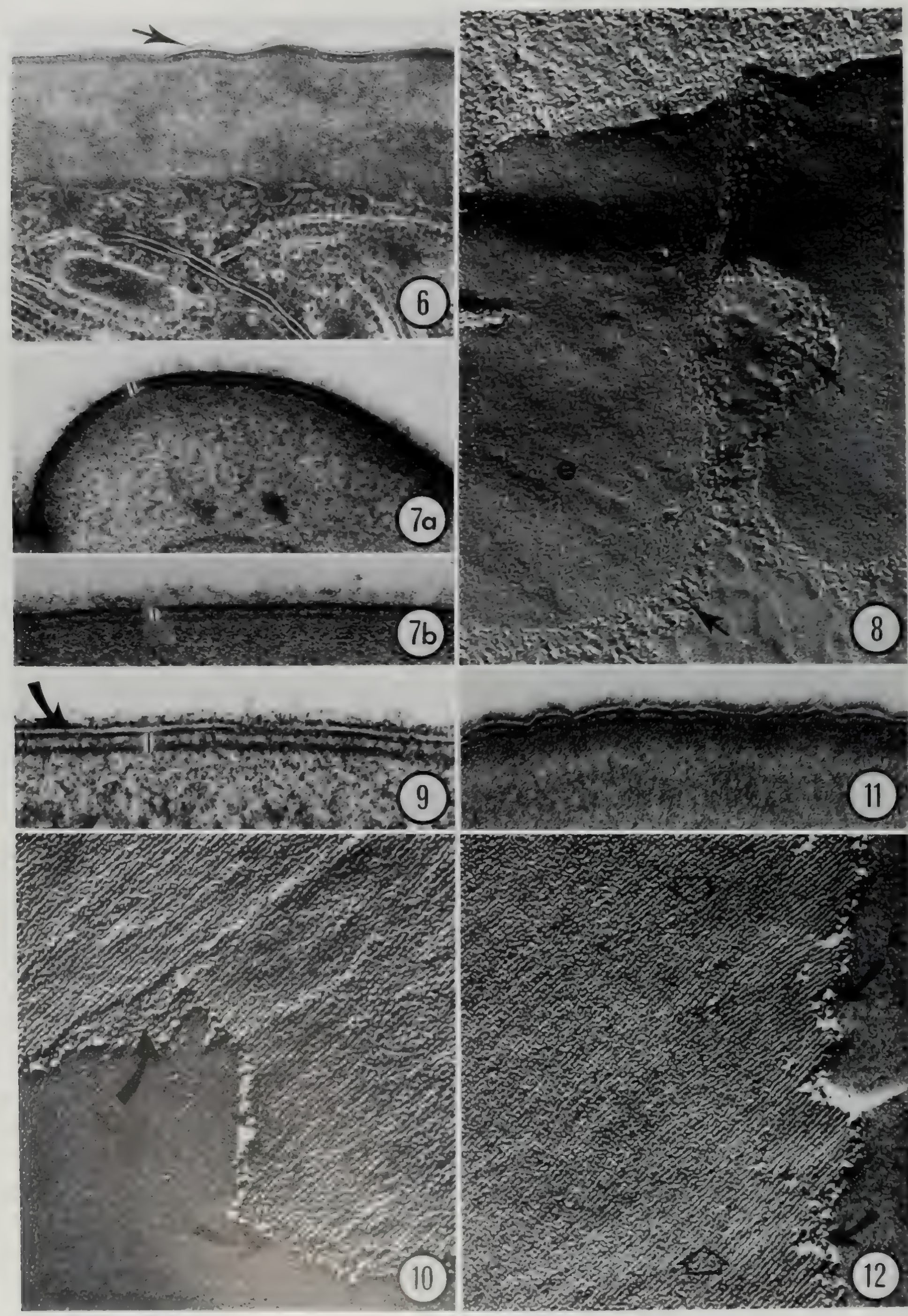
FIGURES 1–5. **1.** Freeze fracture of a glutaraldehyde-fixed larva that was etched following fracture. The initial fracture line (large arrows) through the accessory layer shows filaments protruding from the fracture (small arrow), and etching has exposed its granular surface (line). Note coarse globules or "blisters" in this etch-exposed surface. e = Epicuticle surface. ×94,000. **2.** A thin section of cuticle fixed with glutaraldehyde/ruthenium red. The accessory layer (arrow) is uniformly dense. ×93,000. **3.** Freeze fracture of cuticle fixed with glutaraldehyde/ruthenium red showing the uniform pattern of filaments seen from the inner surface of the accessory layer. ×70,000. **4a.** A thin section of the cuticle surface fixed in glutaraldehyde/tannic acid. Note the trilaminar pattern of the accessory layer (arrow) closely applied to the epicuticle. ×133,000. **4b.** Similar to 4a but showing the accessory layer raised above the epicuticle and having angular bends in it. ×133,000. **4c.** Similar to 4a but showing a cross-banded pattern in the accessory layer where it is obliquely sectioned. ×133,000. **5.** Freeze fracture of cuticle fixed in glutaraldehyde/tannic acid showing the inner surface of the accessory layer. Note irregular clumps of large particles around the margin of the fracture and small poclike spots (arrows) in the filament layer. ×59,000.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Worms fixed in glutaraldehyde and some postfixed in osmium were dehydrated with ethanol according to the standard protocol used for embedding tissues for sectioning. After these ethanol-dehydrated worms were rehydrated, they were freeze fractured. The usual intramembrane fracture planes could not be demonstrated. Although a granular material was identifiable on the cuticle surface, no hint of filament organization of the accessory layer could be found.

Effects of detergents

The effects of the anionic detergent sodium dodecyl sulphate (SDS) and the cationic detergent cetyl trimethylammonium bromide (CTAB) on the accessory layer were determined by both thin sections and freeze fractures. Worms were initially incubated in low concentrations of these detergents (1% SDS and 0.25% CTAB) in HBSS at 37 C with 2 hr of continuous agitation. Thin sections showed that the accessory layer had been modified (Fig. 9). Its outer surface was fuzzy and diffuse, whereas its inner margin, next to the epicuticle remained distinct. Freeze fractures showed fracture patterns similar to those in control worms. That is, the filament layer remained evident and granules were detectable above this (Fig. 10).

In order to see if a critical micellar concentration of these detergents might be found that would completely dissolve the accessory layer, the effects of 10% SDS, and 2%, 5%, and 10% CTAB were examined. None of these had any increased effect on the sectioned appearance of the accessory layer (Fig. 11). Freeze fractures again showed that filaments remained, though in both detergents there may be areas where filaments appeared as rows of globules (Fig. 12). Following 10% CTAB treatment, standard freeze fractures revealed the outer surface of the filament layer for the first time, with only a few adherent granules (Fig. 13). Yet when such worms were etched after fracturing, there was considerable coarsely clumped, irregular material on the surface of the filaments. This suggests that the granules are loosened and can be pulled off in fracturing but do not completely separate as a result of detergent treatment itself.

The accessory layer showed no changes detectable in thin sections as a result of treatment with trypsin for 1.5 hr at 37 C. Similarly, trypsin treatment for 1 hr following 2 hr of incubation in 10% CTAB at 37 C gave no detectable modifications of the accessory layer as examined either in sections or in freeze fractures.

Effect of detergents on viability/infectivity of worms

Worms died in SDS solutions within about 2 hr; however, 95% of the worms remained very active in the CTAB solutions. In fact, in CTAB they appeared hyperactive. It required an incubation time in excess of 5 hr before a significant number of worms died in this detergent. Worms that had been treated with 10% CTAB in HBSS for 2 hr at room temperature were repeatedly washed in HBSS and then were fed to mice by standard infection techniques (250–300 larvae/mouse). Worms were recovered from the intestine 1–2 days later by incubation of the intestine in HBSS. Either no worms or 1–3 worms were retrieved from 6 mice infected in this way. Clearly the detergent treatment rendered the larvae incapable of establishing in the intestine. The

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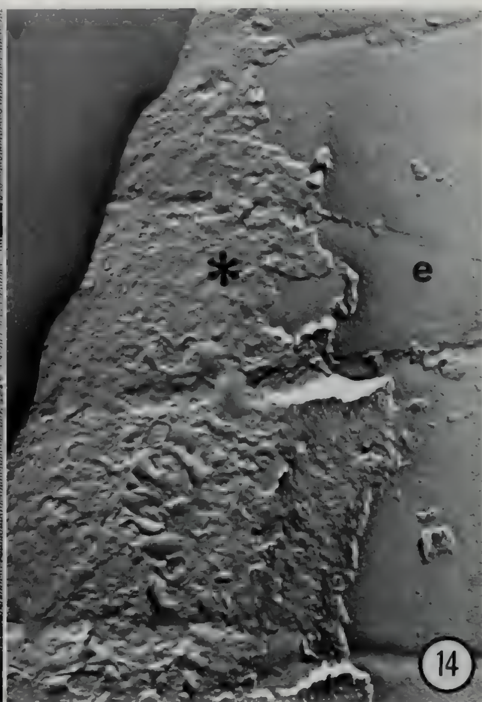
FIGURES 6–12. **6.** Thin section of a larva fixed in glutaraldehyde/osmium and dehydrated in acidified 2,2-DMP. Note that the accessory layer (arrow) is a dense line, whereas the cell and the cytoplasmic membrane are pale from extraction of lipid. ×61,000. **7a, b.** Two thin sections of the cuticle surface fixed in glutaraldehyde, dehydrated with acidified 2,2-DMP, then postosmicated. The accessory layer appears only as fuzzy material on the surface of the epicuticle (lines). a: ×81,000; b: ×97,000. **8.** Freeze fracture of the cuticle of a worm fixed in glutaraldehyde and dehydrated with 2,2-DMP prior to fracturing. Only irregular globules (arrows) are seen lying on top of the epicuticle (e). ×95,000. **9.** Thin section of the cuticle surface of a worm treated with 0.25% CTAB. Note that the outer surface of the accessory layer is diffuse, whereas the inner surface (arrow) is discrete. Epicuticle is denoted by the line. ×136,000. **10.** Freeze fracture of a worm treated as in Figure 9 showing the inner aspect of the accessory layer where filaments appear as rows of globules. Globules from the outer surface extend from the edge of the fractured filaments (arrows). ×125,000. **11.** Thin section of the surface of the cuticle of a worm treated with 10% SDS. Note the diffuse outer surface but discrete inner margin of the accessory layer similar to Figure 9. ×79,000. **12.** Freeze fracture of a 10% SDS-treated worm showing globules of the outer surface of the accessory layer along its fractured edge (arrows), and the transition (open arrows) from filaments to rows of globules on the inner aspect of this layer. ×110,000.



FIGURES 13, 14. **13.** Freeze fracture of the cuticle of a worm treated with 10% CTAB. The outer surface of the filaments of the accessory layer has been exposed. Note the small number of granules adhering to the filaments. ×61,000. **14.** Freeze fracture of the cuticle of a worm rinsed with chloroform/methanol. The accessory layer (asterisk) has been extensively distorted and filaments cannot be resolved. e = Epicuticle surface. ×20,000.

sensitivity of CTAB-treated larvae to 1% pepsin in 1% HCl (a mock stomach environment) was tested. Within about 20 min of submersion of CTAB-treated worms into either pepsin/HCl or 1% HCl (pH ~1.5) in HBSS, many worms were dying and assuming the relaxed shape of dead worms. By 30–45 min, only about 10% of the larvae showed normal activity.

Effects of lipid solvents

Worms were rapidly killed when exposed to a mixture of chloroform and methanol, yet their general internal anatomy remained remarkably intact. Freeze fractures were prepared from worms that had been flushed for about 5 sec with chloroform/methanol, but were quickly rinsed and fixed in glutaraldehyde. In fractures oblique to the cuticle surface, the epicuticle was variably covered by irregular material showing neither filaments nor discrete globules (Fig. 14). In cross fractures of the worms, very few areas of intramembrane surfaces were revealed; the fracture plane passed directly from cell to cell without exposing as many intramembrane areas as would be expected.

When the contents of the 5-sec washes of chloroform/methanol from 10^6 worms were separated by thin-layer chromatography, there was some variation in results. Up to 6 faint spots for neutral lipids were detected with iodine vapors. One of these corresponded to the mobility of the cholesterol standard run at the same time. Phospholipids, separated after the neutral lipids, always showed 1 strong spot close to the solvent front corresponding most closely to the phosphatidylethanolamine control (Fig. 15). Often, a spot corresponding to lysophosphatidylethanolamine was seen when plates were examined immediately (this spot and its control usually faded from the plates). Extracts from larger numbers of worms sometimes gave a hint of other less mobile phospholipids. Chromatograms made from chloroform/methanol rinsed more rapidly over living worms also showed a strong spot cor-

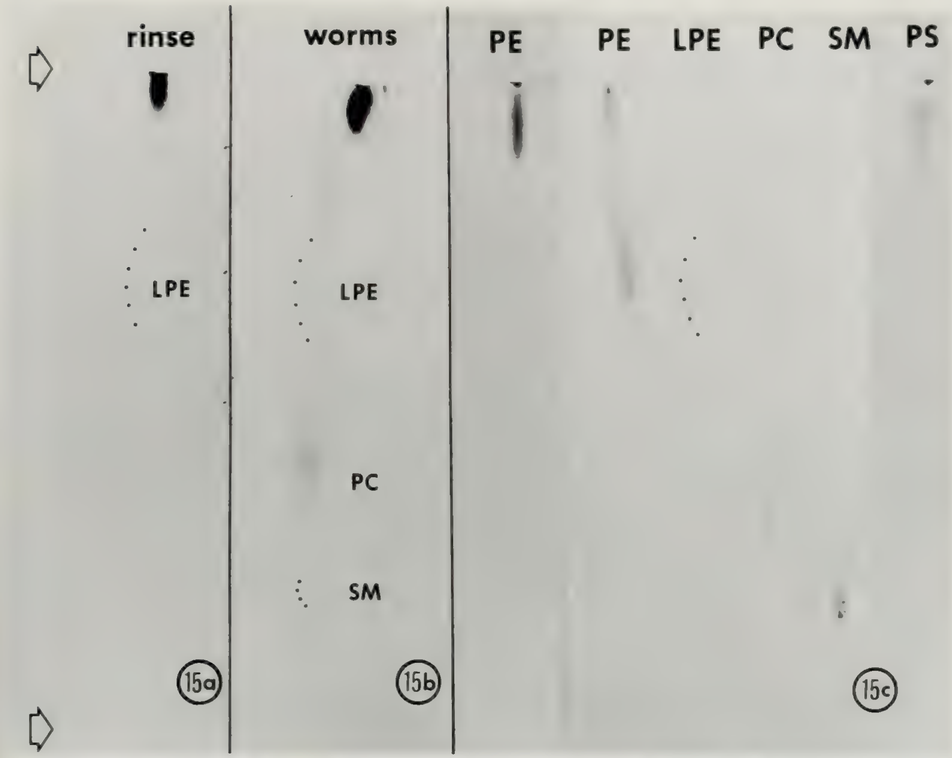


FIGURE 15. Photograph of charred thin-layer chromatograms of phospholipids. **a.** Phospholipid separated from 10^6 living worms by a 5-sec rinse with chloroform/methanol. **b.** Phospholipids isolated from the homogenate of the rinsed worms from a. **c.** Standard phospholipids. The first phosphatidylethanolamine (PE) was from the same plate as shown in a. The second PE was from plate b. The lysophosphatidylethanolamine (LPE) spots faded, but their locations are indicated. PC = phosphatidylcholine, SM = sphingomyelin. The phosphatidylserine (PS) spot is from another plate because this spot was very light in plate b. The solvent front is noted by the upper arrow and the origin by the lower arrow.

responding to phosphatidylethanolamine and a fainter lysophosphatidylethanolamine spot. When surface-rinsed worms or untreated worms were ground and extracted for total lipids, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, and sphingomyelin were detected (Fig. 15). One chromatogram was run of the CTAB and SDS supernatants, but only spots for these detergents were detected.

DISCUSSION

The model proposed by Lee et al. (1984) for the organization of the accessory layer of the cuticle of infective larvae of *Trichinella spiralis* can now be simplified but must also be elaborated upon. Because etching of freeze fractures demonstrates a granular outer surface on the accessory layer, we conclude that the matrix previously thought to surround the globules of the outer layer was only water (ice) that can be removed by the etching process. Hence the number of components in the model of the accessory layer may be reduced from 3 to 2—an external layer of globules and an inner layer of filaments. Freeze-fracture preparations preserve more structural details of the accessory layer than does thin sectioning, where the layer most frequently appears as a single dense line. Worms fixed by either glutaraldehyde or glutaraldehyde and osmium did not retain the globule/filament morphology of the accessory layer when they were dehydrated with ethanol as required for embedding for thin sections, and then were freeze fractured. Clearly, dehydration extracts materials—most likely lip-

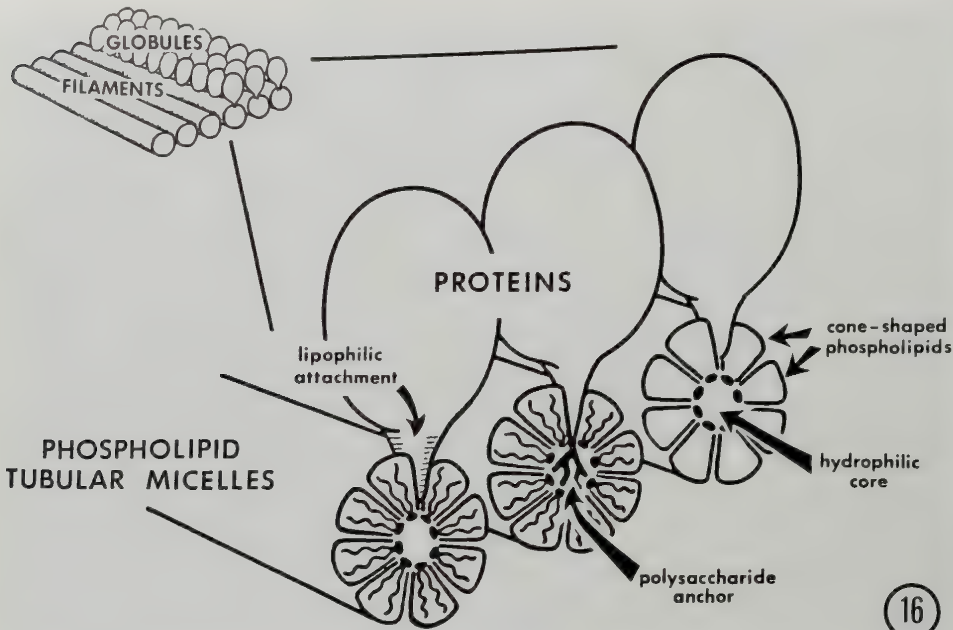


FIGURE 16. A model for the organization of the accessory layer of the cuticle of the infective larva (L_1) of *Trichinella* showing phospholipids arranged as tubular micelles with associated globular proteins. Cone-shaped phospholipids, such as phosphatidylethanolamine, form inverted tubular micelles with their polar groups giving a hydrophilic central core to each micelle. Proteins interact with the micelles by means of a nonpolar, lipophilic attachment moiety. Polysaccharide groups of the 47-kDa proteins may extend through to the hydrophilic core of the micelle. Proteins could be released from the phospholipid micelles by detergents. However, adjacent micelles may interact with each other through their fatty acid sidechains that may require the stronger solvent action of alcohols, acetone, or chloroform to dissociate them. Milder perturbation of the tubular micelles could result in rearrangement of the phospholipids into globular micelles accounting for such patterns after some treatments.

ids. Hence, more importance is given to freeze-fracture observations than to those from thin sections. Our histochemical and chemical extraction experiments allow further conclusions about the composition of the globules and filaments. An updated model is presented in Figure 16.

The layer of globules undoubtedly contains the antigenic surface proteins identified by others (Ortega-Pierres et al., 1984; Silberstein and Despommier, 1984; Pritchard et al., 1985; McLaren et al., 1987). The solubilization of these proteins by detergents (SDS and CTAB) was demonstrated by Pritchard et al. (1985). These detergents have been shown in our thin section and freeze-fracture-etch preparations to remove material from the surface of the accessory layer. However, not all of the globule layer was stripped off by the detergents. The fact that intact larvae of *T. spiralis* did not bind positively charged colloidal iron (and hence lack negative surface charges) and that they did not express PA-TSC-Ag protein-demonstrable carbohydrates on their surface indicates a major difference between the chemistry of the surface of the accessory layer and the surface of the cuticle of many other nematodes. (Negatively charged carbohydrates have been demonstrated as a surface coat of the cuticle on *Haemonchus, Nippostrongylus,* and *Trichostrongylus* by Bone and Bottjer [1985], on *Globodera* by Forrest and Robertson [1986], on *Caenorhabditis, Panagrellus,* and *Meloidogyne* by McClure and Zuckerman [1982] and by Jansson et al. [1986], on *Xiphinema* by Spiegel et al. [1982], and on *Steinernema* by Dunphy and Webster [1987]). Our findings are in agreement with the observation of Ortega-Pierres et al. (1984) that a variety of lectins do not bind with

the surface of intact muscle larvae of *T. spiralis*. Although a cell coat has been shown on the cuticle of adults of *Trichinella spiralis* (Lee et al., 1986), it has not been found to bind lectins (Almond et al., 1986).

Surprisingly, the proteins, as organized on the surface of the muscle larvae, are resistant to a number of proteases. In our studies this included both pepsin and trypsin. Despommier (1983) has also found the accessory layer to be resistant to alpha-chymotrypsin and papain. Stewart et al. (1987), however, reported that the accessory layer could be broken down by *in vitro* treatment with trypsin and bile. They considered this to mimic the intestinal environment and to be important in stimulating the larvae toward further development.

Our study has shown that larvae treated with CTAB remain lively for an extended time *in vitro*, but fail to establish infections when administered by stomach tube to mice. Detergent-washed worms died quickly in a stomach digest solution of pepsin/HCl and also in 1% HCl. It is likely that the *proteins* of the accessory layer allow the larvae to survive passage through the acid environment of the stomach of their host. Lee et al. (1984) and Ortega-Pierres et al. (1984) also suggested that the accessory layer might have this function.

The filament component of the accessory layer was not degraded by pepsin, or trypsin, or by trypsin following removal of some surface proteins by the detergent CTAB. The only reagents that destroyed the organization of the filaments were lipid solvents; ethanol (as in dehydration procedures for thin sections), acetone and methanol (formed *in situ* from 2,2-DMP), or rinses of chloroform and methanol. This indicates a predominantly lipid composition for the filament layer. Thin-layer chromatography of chloroform/methanol rinses of worms demonstrated only traces of neutral lipids. The fact that filipin did not modify the morphology of the filament layer indicates that cholesterol is not a significant component of the filaments. The major phospholipid demonstrated in chromatograms of rinses corresponds most closely to phosphatidylethanolamine, which accounts for 20% of the worm's phospholipids (Castro and Fairbairn, 1969). Because the spot for the control phosphatidylserine overlapped with that of phosphatidylethanolamine, it is possible that it is also present, although it represents only 10% of total phospholipids. It would be expected that rinses of chloroform/methanol could remove some lipids from internal membranes. Likely, the trace amounts of neutral lipids (including cholesterol) and phospholipids did come from internal tissues as fracture planes of internal tissues were found to be modified by the rinses. More complete extraction of homogenates of chloroform/methanol-rinsed worms, or of untreated worms did recover 4 of the 5 phospholipids (phosphatidylethanolamine and lysophosphatidylethanolamine, plus phosphatidylcholine and sphingomyelin) that were identified in *Trichinella* by Castro and Fairbairn (1969).

The dense staining of the accessory layer with ruthenium red may have been due to binding of this dye to phosphatidylethanolamine as demonstrated by Luft (1971). This may further stabilize the phospholipids, giving the very uniform filaments seen in our freeze fractures. On the other hand, tannic acid was found by Kalina and Pease (1977) to fix choline phospholipids in lung surfactant. Perhaps its binding to ethanolamines in accessory layer filaments distorts this lipid's morphology, giving rise to pocs and disorientation of the surface protein globules in its freeze-fractured patterns.

The appearance of filaments may be accounted for by the tendency of ethanolamine phospholipids to form tubular micelles, rather than bilayers, as do the choline phospholipids (DeKrujiff et al., 1981). Filament-like patterns have been demonstrated in freeze fractures of liposomes of cardiolipin (found in the inner mitochondrial membrane) by Vail and Stollery (1979). The geometry of the phospholipid determines the orientation of the phospholipid in the micelle; conical phospholipids (including phosphatidylethanolamine) orient with their polar group inwards forming a hydrophilic core and a hydrophobic outer surface, and inverted cone phospholipids orient with their polar groups outward, giving a reversed hydrophilic/hydrophobic arrangement. Interactions between adjacent filaments and between filaments and proteins will likely vary in these 2 conditions. It is most likely that the filaments of the accessory layer of the cuticle of *T. spiralis* are of the first form, with a hydrophilic core. Proteins would be associated with the filaments by means of a lipophilic moiety inserted between the acyl chains of the phospholipids. The 47-kDa surface protein, which has been demonstrated to have a lectin-binding site (presumably carbohydrate) only when isolated from the worm's surface (Parkhouse et al.,

1981; Ortega-Pierres et al., 1984), may be anchored into the filaments by this component that extends into the hydrophilic core of the lipid micelles. The proteins could be released from the tubular micelles by detergents, but apparently detergents cannot solubilize the tubular micelles. This may be due to strong lipid–lipid interactions between adjacent micelles that require lipid solvents such as alcohols, acetone, or chloroform to break them.

In summary, the cuticle of infective larvae of *Trichinella spiralis* is covered by a complex accessory layer that appears to consist of an outer layer of globular proteins attached to an inner layer of phospholipid micelles. The external proteins lack exposed sugars or negative charges. They are recognized as antigens by hosts but may be equally or more important as buffers against the acidity of the hosts' stomach environment. The organization of phospholipids as tubular micelles may be unique in animal surfaces.

ACKNOWLEDGMENTS

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A COMPARISON OF *GIARDIA MICROTI* AND *SPIRONUCLEUS MURIS* CYSTS IN THE VOLE: AN IMMUNOCYTOCHEMICAL, LIGHT, AND ELECTRON MICROSCOPIC STUDY

Mary M. Januschka, Stanley L. Erlandsen, William J. Bemrick, Daniel G. Schupp, and Dennis E. Feely*

Departments of Cell Biology and Neuroanatomy, and Veterinary Pathobiology,
Schools of Medicine and Veterinary Medicine, University of Minnesota,
Minneapolis, Minnesota 55455

ABSTRACT: We have shown that cysts of the genus *Spironucleus* share many common morphological features with *Giardia* cysts including: 2–4 nuclei, flagellar axonemes, a distinct cyst wall, and they even display the same immunostaining as *Giardia* cysts when labeled with antibodies specific for *Giardia* cyst wall. A direct comparison of *Spironucleus muris* and *Giardia microti* cysts has revealed that cysts of *S. muris* are significantly smaller than cysts of *G. microti*. At the ultrastructural level, the cyst walls are similar in fibrillar appearance, but the width of the *S. muris* cyst wall is significantly less than that of *G. microti*. The cysts of *S. muris* also differ from *G. microti* in that they contain a striated rootlet fiber, flagellar sheath, and numerous glycogen rosettes. Characteristic features of *Giardia* include the adhesive disc and median body. Although the cysts of *Spironucleus* and *Giardia* are similar in appearance, these unique morphological features can be used to distinguish between the 2 protozoa and should be employed in the detection of *Giardia* cysts in water samples.

Giardiasis is the most common waterborne intestinal disease caused by an enteric protozoon in the United States (Centers for Disease Control, 1978). Transmission occurs through the ingestion of *Giardia* cysts, usually from either untreated or inadequately treated water (Craun, 1979). Methods have been developed to recover *Giardia* cysts from water samples based on cyst size (Jakubowski, 1984a). Recognition of the recovered *Giardia* cysts relies on light microscopic detection of morphological characteristics, immunofluorescent labeling of cyst antigens, or both (Jakubowski, 1984a; Sauch, 1985; Sorenson et al., 1986).

The genus *Giardia* belongs to the order Diplomonadida and the family Hexamitidae. *Spironucleus* also belongs to this family. Unlike the trophozoites that have distinctive morphology, the cysts of *Giardia* and *Spironucleus* are quite similar in shape. The cysts of *Giardia* and *Spironucleus* are oval and are surrounded by a 0.3–0.5-μm-thick filamentous wall (Brugerolle et al., 1973; Kulda and Nohynkova, 1978). Both *Giardia* and *Spironucleus* cysts contain 2–4 nuclei and flagellar axonemes. The *Spironucleus* cyst is $7.4 \pm 0.3 \times 4.0 \pm 0.2$ μm (Kunstyr, 1977), whereas the *Giardia* cyst is reported to be slightly larger, measuring $8–12 \times 7–10$ μm (Kulda and Nohynkova, 1978).

Little is known about the fine structure of *Spironucleus* cysts, but numerous reports exist on the ultrastructure of *Giardia* cysts of murine and human origin (Sheffield and Bjorvatn, 1977; Nemanic et al., 1979; Owen et al., 1979; Luchtel et al., 1980; Feely et al., 1984). In this paper, we compare the structural and immunocytochemical similarities and differences between *Spironucleus* and *Giardia* cysts by using Nomarski differential interference microscopy (DIC), immunofluorescence, and transmission electron microscopy. The information derived should provide additional criteria useful for the differentiation of cyst forms, other than those relying only on size and shape.

MATERIALS AND METHODS

Prairie voles (*Microtus ochrogaster*) that were infected with *Giardia microti* also were found to contain *Spironucleus muris*. The *G. microti* and *S. muris* cysts were isolated from the feces by the zinc sulfate flotation procedure of Faust et al. (1970).

For electron microscopy, the *S. muris* and *G. microti* cysts were fixed overnight in 5% glutaraldehyde and 0.5% paraformaldehyde, in 0.1 M phosphate buffer, pH 7.3. Albumin was then added to the cysts according to the method of Heusser and Van de Velde (1981) to encapsulate the specimen. The cysts were postfixed for 1 hr in 1% osmium tetroxide and 1.5% potassium ferricyanide. The samples were rinsed 2 times, 10 min each time, in 0.1 M phosphate buffer, and then transferred back into the same osmium ferricyanide fixative for an additional hour (Langford and Coggeshall, 1980). After osmication, the cysts were rinsed in 0.1 M phosphate buffer, dehydrated through a series of graded

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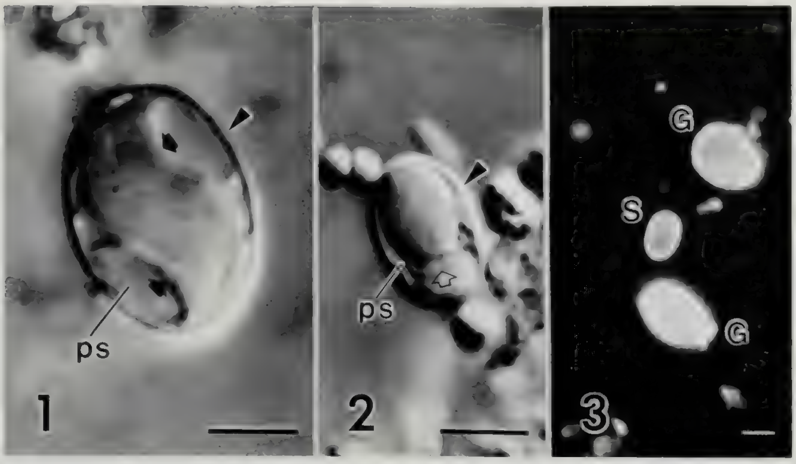
* Department of Oral Biology and Basic Science, University of Nebraska Medical Center, Lincoln, Nebraska 68583.



FIGURES 1–3. **1.** A Nomarski DIC micrograph of a *G. microti* cyst. The sigmoidal appearance is typical of "binary" *Giardia* cysts. Characteristic features of the "binary" cyst include 2 adhesive discs (solid arrow), a well-defined cyst wall (arrowhead), and a conspicuous peritrophic space (ps). Bar = 5 μm. **2.** A Nomarski DIC micrograph of an *S. muris* cyst at the same magnification as Figure 1 illustrates the size difference between *Spironucleus* and *Giardia* cysts. Typical features of *S. muris* cysts by light microscopy include a distinct cyst wall (arrowhead), a narrow peritrophic space (ps), and flagella in the peritrophic space encircling the trophozoite open arrow). Bar = 5 μm. **3.** Immunofluorescence staining of a polyclonal antibody (Riggs et al., 1983) specific for *Giardia* cysts. Intense immunostaining of both *S. muris* (S) and *G. microti* (G) cysts demonstrates their immunological similarity. Bar = 5 μm.

ethanols, propylene oxide, and embedded in Epon 812. Thin sections were cut on an LKB-Huxley ultramicrotome. The sections were stained for 20 min in saturated uranyl acetate in 1% acetic acid (Erlandsen and Chase, 1972), followed by 2–5 min in lead citrate (Reynolds, 1963). The grids were examined with a JEOL-100CX electron microscope.

Using light microscopy, the length and width of *G. microti* and *S. muris* cysts were measured using an eyepiece reticule calibrated with a stage micrometer on an Olympus BH2 microscope equipped with Nomarski differential interference contrast (DIC) optics.

The thickness of the cyst wall was measured from electron micrographs. Two perpendicular lines, representing the major and minor axes, were drawn on each cyst, dividing it into 4 quadrants. The thickness of the filamentous layer of the cyst wall was determined at each intercept and an average thickness was calculated for each cyst. A total of 17 cysts was measured for both *S. muris* and *G. microti* to determine the width of the cyst wall.

Immunocytochemical studies were performed on *Giardia* cysts obtained from the vole (*Microtus ochrogaster*), muskrat (*Ondatra zibethica*), mouse (*Mus musculus*), beaver (*Castor canadensis*), dog (*Canis familiaris*), and human (*Homo sapiens*). *Spironucleus* cysts were only obtained from the vole. Both *Giardia* and *Spironucleus* cysts were attached to glass slides with poly-L-lysine. Five antibodies directed against *Giardia* cysts were tested. Direct immunofluorescence was used with mouse monoclonal and guinea pig polyclonal anti-*Giardia* antibodies (Riggs et al., 1983). The unlabeled antibody enzyme method was used, as previously described (Erlandsen et al., 1975), to localize rabbit antisera to *Giardia* cysts of murine or human origin. It was also used with a guinea pig antiserum that had been produced in our laboratory, which was directed against *G. muris* cysts. Immunofluorescent antibody-stained slides were examined on an Olympus BH2 epifluorescence microscope at an excitation wavelength of 450–490 nm, and Nomarski DIC or bright-field optics were used to examine slides stained with the antibody–enzyme method.

RESULTS

Two types of *G. microti* cysts were observed using light microscopy. They corresponded to either the "single individual cyst" (2 nuclei) or the "binary cyst" (4 nuclei) described by Boeck (1917, 1919) and more recently by Feely (1987). Over 68.5 ± 15.1% (n = 14) of the *G. microti* cysts detected in feces were of the binary type, and therefore, only this type of *Giardia* cyst was used for comparative measurements with cysts of *S. muris*. Examination of binary *Giardia* cysts with Nomarski DIC revealed the presence of a distinct cyst wall surrounding 2 S-shaped trophozoites (Fig. 1). The trophozoites contained 2 nuclei, intracellular axonemes, assembled adhesive discs, and were often seen separated from the cyst wall by the peritrophic space. The av-

TABLE I. *Immunoreactivity of* Giardia *and* S. muris *cysts.*

Anti-*Giardia* antibodies tested	Source of *Giardia* cysts*						*S. muris* cyst
	Dog	Bv	Man	Mouse	Mk	Vole	Vole
Mouse monoclonal (obtained from Dr. J. L. Riggs)†	+	+	+	0	0	0	0
Guinea pig polyclonal (obtained from Dr. J. L. Riggs)†	+	+	+	+	+	+	+
R-GLID (obtained from Dr. J. Sauch)†	+	+	+	+	+	+	+
R-whole cyst (obtained from Dr. J. Sauch)†	+	+	+	+	+	+	+
Guinea pig anti-*Giardia* cyst‡	+	+	+	+	+	+	+

* Bv, beaver; Mk, muskrat; + = positive immunostaining; 0 = negative immunostaining.
† Produced against *G. lamblia* cysts.
‡ Produced against *G. muris* cysts.

erage size of the binary *G. microti* cysts was $14.5 \pm 2.0 \times 9.5 \pm 0.8$ μm (n = 20). Very few intracellular organelles were observed in the cyst form of *S. muris* when examined with Nomarski DIC microscopy (Fig. 2). A distinct cyst wall and flagellar axonemes surrounding a central cytoplasmic area were easily discernible. No nuclei or other intracellular organelles were detected. The size of *S. muris* cysts was considerably smaller than *G. microti* cysts, measuring $8.3 \pm 1.3 \times 5.2 \pm 1.1$ μm (n = 20).

Immunocytochemical analysis revealed that all *Giardia* cysts were intensely stained with each of the antibodies tested, except for the mouse monoclonal antibody obtained from Riggs et al. (1983). This antibody did not stain *Giardia* cysts from the muskrat (*G. ondatrae*), mouse (*G. muris*), or the vole (*G. microti*) (Table I). The same pattern of positive and negative immunostaining was observed with cysts of *Spironucleus muris* (see Table I). All antibodies that positively stained *G. microti* cysts also produced positive staining of *S. muris* cysts (Fig. 3).

The ultrastructural features of *G. microti* and *S. muris* cysts are illustrated in Figure 4. The binary *G. microti* cyst contains 2 mature trophozoites, each of which possesses a complete ventral adhesive disc, and a pattern of cytoplasmic organelles. The *S. muris* cyst is recognizable by the presence of the flagellar sheath, and the caudal flagella which are partially enveloped by a striated rootlet fiber (Figs. 4, 5).

Examination of *S. muris* cysts occasionally revealed 1–2 nuclei and in one instance, 3 nuclei were encountered. Also, rosettes of glycogen were seen in association with the caudal flagella of *S. muris*, whereas glycogen rosettes were infrequently seen within the cysts of *G. microti*.

The trophozoites within both *G. microti* and *S. muris* cysts are surrounded by a peritrophic space and a filamentous cyst wall (Figs. 4–6). Flagella are frequently seen in the peritrophic space and may be observed more often in *S. muris* cysts than in *G. microti* cysts. The cyst walls of both *S. muris* and *G. microti* appear similar in their structure and are composed of fine filaments attached to underlying membranes. However, the filamentous layer of the cyst wall is thicker in *G. microti* cysts, being 0.194 ± 0.031 μm (n = 17), whereas the filamentous layer of *S. muris* cysts is 0.148 ± 0.018 μm (n = 17).

DISCUSSION

There is a striking resemblance between *Spironucleus* and *Giardia* cysts at the light microscopic and the ultrastructural level. The similarities between cysts of *S. muris* and *G. microti* from the vole are listed in Table II. One would expect to find these similarities because both are from the same family, *Hexamitidae*. In addition to these morphological characteristics, the immunoreactivity of *S. muris* and *G. microti* cysts is identical when tested with antibodies produced against *G. muris* or *G. lamblia* cysts.

Distinct differences are detectable between the cysts of *S. muris* and *G. microti*, particularly at the ultrastructural level (Table II). These differences include size, cyst wall thickness, and the presence or absence of characteristic intracellular organelles.

The *S. muris* cysts are approximately 40–50% smaller ($P < 0.0005$) than *G. microti* cysts. The filamentous layer of the *S. muris* cyst wall is approximately 25% thinner than the *G. microti* cyst wall, and although the difference is slight, it is significant ($P < 0.0005$). The appearance of the cyst walls of *S. muris* and *G. microti* suggests that their structure is similar and this is supported by their immunological cross-reactivity.

Intracellular organelles are present within *G.*

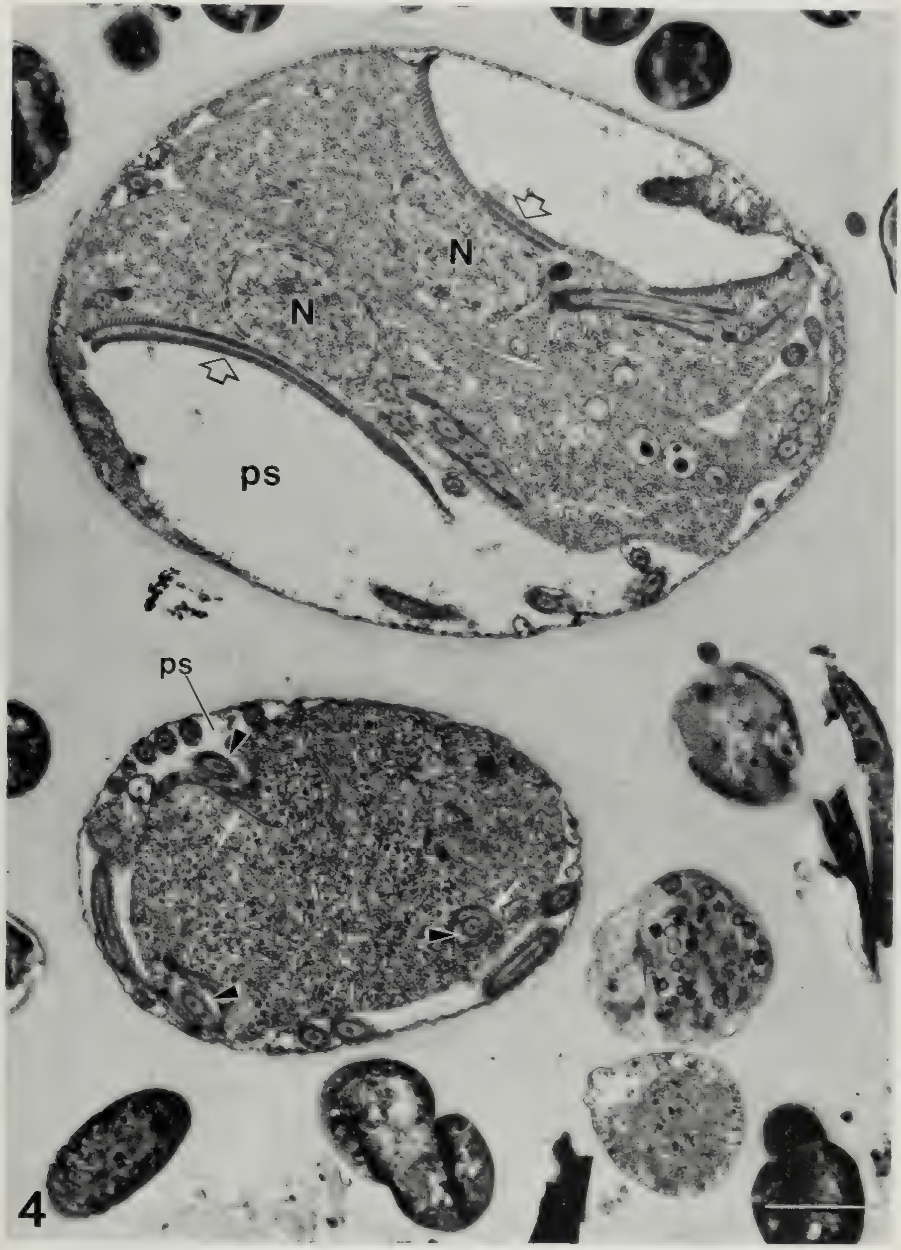


FIGURE 4. A transmission electron micrograph of a *Giardia microti* cyst and a *Spironucleus muris* cyst illustrating the size difference between the 2 cysts and their characteristic features. Structures unique to "binary" *Giardia* cysts include 2 adhesive discs (open arrow) associated with 2 separate trophozoites, and a prominent peritrophic space (ps). The *Spironucleus muris* cyst is characterized by the striated rootlet fiber (white arrow) and flagellar sheath (arrowhead), and numerous profiles of flagella within the narrow peritrophic space (ps). N = nucleus. Bar = 1 μm.


N
ps
5
ps
G
S
ps
6

microti cysts, but not in *S. muris* cysts; these include elements of the adhesive disc and the median body. The adhesive disc, which is part of the attachment organelle, is found only in this genus and can be recognized at the light microscopic and ultrastructural level. The median body, whose function remains undetermined, can be detected within *Giardia* cysts at the light microscopic and ultrastructural levels (Sheffield and Bjorvatn, 1977; Owen et al., 1979). However, we did not observe this organelle within cysts examined in this study.

Intracellular organelles, present within *S. muris* cysts but not observed in *G. microti,* include a striated rootlet fiber, flagellar sheath, and abundant glycogen rosettes. The striated rootlet fiber and flagellar sheath are specializations associated with the intracellular course of the caudal flagellar axonemes (Erlandsen and Chase, 1972). The rosettes of glycogen are frequently observed in association with the intracellular axonemes of the caudal flagella in *S. muris* from the vole and also in *S. muris* from the mouse, rat (Erlandsen and Chase, 1972; Brugerolle et al., 1973; Brugerolle, 1974), and in *Hexamita intestinalis* from the frog (Erlandsen, unpubl. obs.).

Detection of *Giardia* cysts in water samples by light microscopy requires observing an object of the right size and shape and identification of at least 2 of the following 3 features of *Giardia* cysts: (1) the presence of 2–4 nuclei; (2) axonemes; and (3) the median body (Jakubowski, 1984a; Sauch, 1985). *Spironucleus* cysts also contain the first 2 organelles listed; therefore their differentiation from *Giardia* cysts will be dependent upon recognition of either elements of the adhesive disc or the median body, if present in *Giardia* cysts. Immunofluorescence localization of *Giardia* antigens has been used in detecting *Giardia* cysts in water samples (Jakubowski, 1984b; Sauch, 1985; Sorenson et al., 1986). Our results indicated that none of the antibodies tested to date against *Giardia* were specific enough to distinguish between *Giardia* and *Spironucleus* cysts. Therefore, to prevent confusion in cyst recognition and an overestimation of *Giardia* cyst density within a sample, immunofluorescence should be used in combination with light microscopic methods for the identification of *Giardia* cysts as suggested by Sauch (1985).

The detection of *Spironucleus* cysts in water samples has not yet been reported, but the large distribution of this parasite in animals inhabiting water environments (i.e., fish, reptiles, amphibians, mammals, and birds) (Kudo, 1939; Hamajima and Ishii, 1963; Kulda and Lom, 1964; Kulda and Nohynkova, 1978) as well as the presence of the protozoon in invertebrates and also free-living forms (Kudo, 1939; Mackinnon and Hawes, 1961) has suggested their presence and indicated a need for some caution when examining water samples for the presence of *Giardia* cysts.

TABLE II. *A comparison of* Spironucleus muris *and* Giardia microti *cysts from the vole (*Microtus ochrogaster*).*

Cyst characteristics	*Spironucleus muris*	*Giardia microti*
Similarities		
2–4 nuclei	+	+
Axonemes	+	+
Peritrophic space	+	+
Immunoreactivity*	+	+
Differences		
Length (n = 20)	8.3 ± 1.3 μm	14.5 ± 2.0 μm
Width (n = 20)	5.2 ± 1.1 μm	9.5 ± 0.8 μm
Adhesive disc	–	+
Median body		+
Striated rootlet fiber	+	–
Flagellar sheath	+	–
Glycogen rosettes	+++ (caudal flagella)	+
Wall thickness (n = 17)	0.148 ± 0.018 μm	0.194 ± 0.031 μm

* Identical immunofluorescent staining (either both positive or both negative) with antibodies directed against *G. muris* or *G. lamblia* cysts.

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FIGURES 5, 6. **5.** A transmission electron micrograph of a *Spironucleus muris* cyst. Unique features include the striated rootlet fiber (white arrow) and flagellar sheath (arrowhead). N = nucleus, ps = peritrophic space. Bar = 0.5 μm. **6.** A transmission electron micrograph showing a comparison of the cyst walls of both *G. microti* and *S. muris*. The cyst wall (arrowhead) of *Giardia* (G) is slightly thicker than that of *Spironucleus* (S), but both cyst walls are composed of a network of interwoven fibrils (see arrows in insets for each cyst). ps = Peritrophic space; white arrow = striated rootlet fiber. Bar = 0.5 μm, insets = 0.1 μm.

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RHINEBOTHRIUM DEVANEYI N. SP. (EUCESTODA: TETRAPHYLLIDEA) AND *ECHINOCEPHALUS OVERSTREETI* DEARDORFF AND KO, 1983 (NEMATODA: GNATHOSTOMATIDAE) IN A THORNY BACK RAY, *UROGYMNUS ASPERRIMUS*, FROM ENEWETAK ATOLL, WITH PHYLOGENETIC ANALYSIS OF BOTH SPECIES GROUPS

Daniel R. Brooks and Thomas L. Deardorff
Department of Zoology, University of British Columbia, 2075 Westbrook Mall,
Vancouver, British Columbia, Canada V6T 2A9, and
Fishery Research Branch, United States Food and Drug Administration, Box 158,
Dauphin Island, Alabama 36528

ABSTRACT: The new species is a member of an apparently monophyletic group within the genus that includes *R. flexile*, *R. walga*, *R. himanturi*, *R. burgeri*, *R. euzeti*, *R. hawaiiensis*, *R. urobatidium*, *R. paratrygoni*, *R. ditesticulum*, *R. tetralobatum*, *R. margaritense*, *R. biorchidum*, and *R. spinicephalum*. All of these species have bothridia with medial longitudinal septa, a constriction at mid-bothridium, and, primitively, at least 42 loculi per bothridium and 17–22 testes per proglottid. Of the above, the new species is apparently most closely related to *R. burgeri*, with which it shares an increased number of testes (30–43) per proglottid, a V-shaped ovary, and a muscular genital pore. The new species is distinct by virtue of possessing 94–152 loculi per bothridium—no other known species has more than 78. This is the second report of *Echinocephalus overstreeti* from a stingray. It represents a new host, *U. asperrimus*, and a new location, Enewetak Atoll. Phylogenetic and biogeographic analysis of each species group suggests an ancient Tethys Sea–circum-Pacific origin and evolution. This supports the hypothesis of ancient Pacific origins for potamotrygonid stingrays.

In 1981, the Bernice P. Bishop Museum (BPBM), Honolulu, conducted an expedition to Enewetak Atoll to study marine vertebrates and invertebrates. During this survey, the gastrointestinal tract (specific location not noted) of a thorny back stingray, *Urogymnus asperrimus* (Bloch and Schneider), was found to contain 8 specimens of a previously undescribed tetraphyllidean cestode and 7 adult nematodes. Worms were removed from the gastrointestinal tract of the captured host by the scientists of the BPBM, placed in formalin, and stored in ethyl alcohol. Subsequently, the cestodes were stained with acetocarmine and mounted in Permount for study as whole mounts. Nematodes were cleared and examined in lactic acid. Average measurements of characters are presented with ranges and sample size (n =) in parentheses. Measurements are in μm unless stated otherwise. Figures were drawn with the aid of a drawing tube. Scanning electron microscopy was used to verify morphology of scolex.

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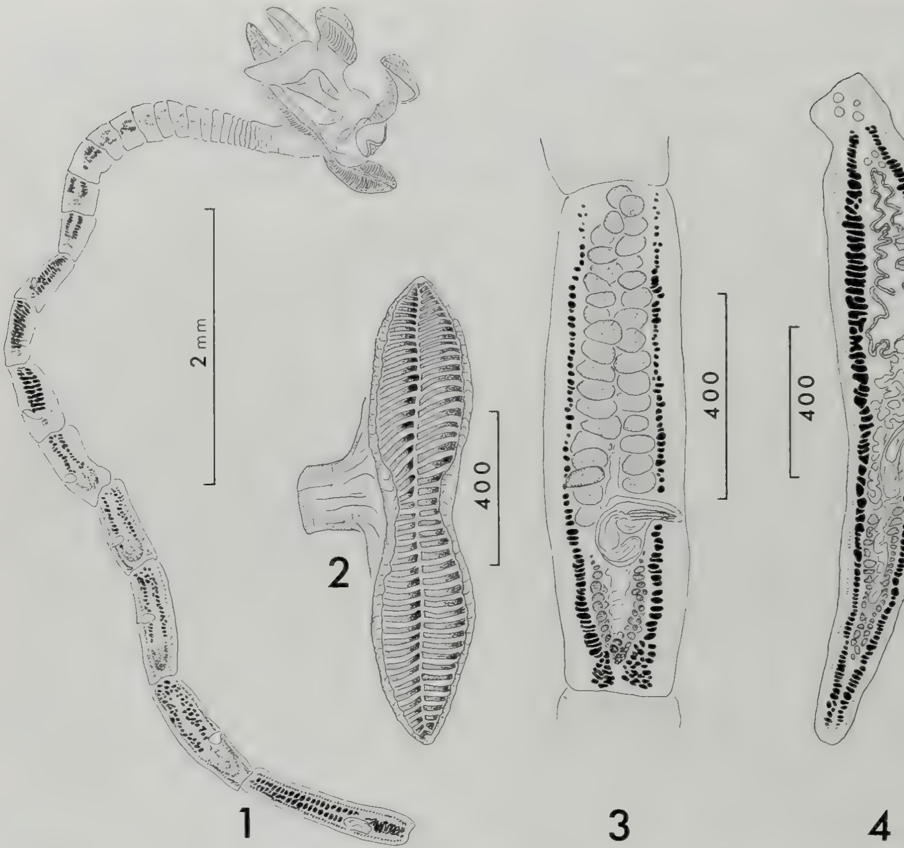
DESCRIPTION

Rhinebothrium devaneyi n. sp.
(Figs. 1–4)

General description (based on 8 specimens): Strobila 0.9 (0.5–1.2) cm long (n = 5), acraspedote, apolytic, composed of 33 (26–39) proglottids (n = 5). Scolex aspinose, composed of 4 pedicellated, elongate, septate bothridia. Bothridia with margins curled and lobulated, divided longitudinally by median septum, with slight constriction between lobes; total number of loculi per bothridium 128 (94–152) (n = 8) plus terminal loculus at tip of each lobe. Bothridial lobes 1.0 (0.9–1.2) mm long by 252 (119–320) wide (n = 4). Pedicels 199 (62–288) long (n = 7). Cephalic peduncle short, 286 (196–445) long (n = 5). Immature proglottids wider than long. Mature proglottids 0.8 (0.5–1.9) long by 287 (241–356) wide (n = 14). Genital pore 60% (50–66%) of proglottid length from anterior end (n = 20), irregularly alternating.

Male genitalia: Testes 37 (30–43) in number (n = 20), 18 (14–21) poral, 19 (15–22) aporal (n = 14). Cirrus sac elongate, 197 (137–240) long by 91 (65–116) wide (n = 13), containing spined eversible cirrus. Genital atrium shallow, genital pore highly muscular.

Female genitalia: Vagina anterior to cirrus sac, sphincter present. Ovary X-shaped in cross section, V-shaped in frontal view; lobes extending anteriorly to posterior margin of cirrus sac; 240 (112–471) long by 93 (36–222) wide at isthmus (n = 14). Vitellaria along lateral margins and extending entire length of proglottid, not in single file, converging toward midline of proglottid at posterior extremity, follicular, 1.0 (0.4–



FIGURES 1–4. *Rhinebothrium devaneyi* n. sp. **1.** Entire worm. **2.** Bothridium (diagrammatic). **3.** Mature proglottid. **4.** Terminal proglottid.

1.7) mm long (n = 16); follicles elliptical, 18 (11–89) in diameter (n = 30). Uterus simple, median, tubular (n = 1). Eggs not seen.

TAXONOMIC SUMMARY

Twenty of the species of *Rhinebothrium,* including *R. devaneyi,* are characterized by having a median longitudinal septum on the face of each bothridium (6 species of *Caulobothrium* share this trait as well): *R. monodi* Euzet, 1954; *R. baeri* Euzet, 1956; *R. maccallumi* Linton, 1924; *R. tumidulum* (Rudolphi, 1819) Euzet, 1956; *R. setiensis* Euzet, 1955; *R. lintoni* Campbell, 1970; *R. burgeri* Baer, 1948; *R. flexile* Linton, 1890; *R. himanturi* Williams, 1964; *R. walga* (Shipley and Hornell, 1905) Euzet, 1956; *R. euzeti* Williams, 1958; *R. hawaiiensis* Cornford, 1974; *R. urobatidium* (Young, 1955) Appy and Dailey, 1978; *R. paratrygoni* Rego and Dias, 1976; *R. ditesticulum* Appy and Dailey, 1978; *R. margaritense* Mayes and Brooks, 1981; *R. spinicephalum* Campbell, 19[illegible] *[illegible]batum* Brooks, 1977; *R. biorchidum* Huber and Schmidt, 1985; and *R. devaneyi.* This group also includes all but 2 of the members of the genus having more than 40 loculi per bothridium. The last 14 species listed above possess bothridia that are constricted in the middle (see Fig. 2). Of these, *R. flexile, R. burgeri,* and *R. devaneyi* have ovaries that appear V-shaped in frontal view rather than H-shaped, because of the posterior confluence of the ovarian lobes and the presence of an indistinct ovarian isthmus. *Rhinebothrium devaneyi* most closely resembles *R. burgeri* by having 30–43 testes per proglottid (the rest of the "constricted" species have 17–22 or fewer) and by having a muscularized genital pore associated with a shallow genital atrium. The new species differs by having a mean number of 128 loculi per bothridium, more than double that reported for *R. burgeri* (50) and at least 50 more than any other known species (*R. euzeti* has 78, *R. paratrygoni* has 72–76, and *R.* sp. of Williams [1966] has 76).

Rhinebothrium biorchidum was reported by Huber and Schmidt (1985) to have a V-shaped ovary. We examined the type specimens (USNM Helm. Coll. Nos.

77889, 77890) and determined that only 1 proglottid, the terminal segment which they illustrated as figure 4, had a V-shaped ovary. The ovary in the terminal proglottid of most species belonging to *Rhinebothrium,* however, appears V-shaped because of extreme attenuation of the proglottid. This is the case with *R. biorchidum.* The ovaries in most proglottids of *R. biorchidum* are H-shaped. The placement of this species in our cladogram reflects this finding.

Specimens deposited: Holotype, USNM Helm. Coll. No. 79815; Paratypes, USNM Helm. Coll. No. 79876, University of Nebraska State Museum No. 20970, BPBM No. 1981.334.

Host: Urogymnus asperrimus (Bloch and Schneider) thorny back ray (Dasyatididae); BPBM cat. No. 27792, J. E. Randall and D. M. Devaney, 12 July 1981.

Site of infection: Recovered from gastrointestinal tract (?spiral valve).

Locality: Enewetak Atoll, in near shore lagoon.

Etymology: This species is named in honor of the late curator of the Invertebrate Zoology Collection at BPBM, Dr. Dennis M. Devaney.

REMARKS

There is disagreement about the phylogenetic utility and stability of various traits used to characterize species of *Rhinebothrium*; nevertheless, all taxonomists studying the group resort to a consistent set of traits in their descriptive work. At present, there is no phylogenetic hypothesis for the group, so disagreements have no data-based frame of reference. Phylogenetic systematics (Hennig, 1966; see Brooks, 1985, for a review of methods and uses published in the journal) embodies a reproducible method for analyzing data according to genealogical criteria. The cladogram, or phylogenetic tree, that results from such studies represents the most parsimonious estimate of phylogeny allowed by the data at hand. The robustness of such hypotheses can be tested by adding new data, or by finding novel evolutionary insights implied by the relationships shown on the tree. We have provided a phylogenetic tree for 20 of the species of *Rhinebothrium.*

The group *R. flexile* + *R. burgeri* + *R. devaneyi* is postulated to be the sister-group of the other 11 species having constricted bothridia. Those 10 are postulated to form a monophyletic group characterized by the possession of relatively long bothridial pedicels (at least half as long as the bothridia). Of this group, *R. himanturi* is postulated to be the sister-species of the other 9 because they all have 11–13 or fewer testes per proglottid, whereas *R. himanturi* has 19–20, similar to *R. flexile* (17–22) and *R. setiensis* (25–35). Of the remaining 9 species, *R. walga* and *R. hawaiiensis* appear to share the unique trait of having longitudinal bands of vitellaria rather than discrete follicles. *Rhinebothrium euzeti* is distinct by virtue of having 78 loculi per bothridium, but does not share any derived traits with any of the other 8 members of the group. The last 6 species form a group, recognized by Brooks et al. (1981a), characterized by having strongly craspedote proglottids, more than 50 proglottids per strobila, squared proglottids rarely longer than wide, and an average of 8 or fewer testes per proglottid. Brooks et al. (1981a) presented a cladogram for these 6 species (*R. margaritense* Brooks and Mayes, 1980, was listed as *R.* sp.), but considered a low number of bothridial loculi to be the primitive trait for the group. Comparisons with the rest of the species considered herein suggest that 47–56 loculi per bothridium is the plesiomorphic condition. This changes the placement of *R. ditesticulum* but does not alter any of the coevolutionary or biogeographic conclusions drawn by Brooks et al. (1981b), which are discussed later.

The phylogenetic hypothesis for 20 of the species belonging to *Rhinebothrium* (Fig. 5) and discussed above is based on a suite of 27 anatomical characters presented in the figure legend. We have confirmed these characters for 12 of the species by examination of type material or voucher specimens (*R. maccallumi, R. devaneyi, R. flexile, R. hawaiiensis, R. lintoni, R. urobatidium, R. paratrygoni, R. margaritense, R. ditesticulum, R. tetralobatum, R. biorchidum,* and *R. spinicephalum.* Note: There are more species of *Rhinebothrium.*) We have not included a character for the true hinged condition because we are unsure, based on original descriptions, which species really have this characteristic.

Figure 5 is the cladogram summarizing the putative phylogenetic relationships discussed above. It is evident that although every described species of *Rhinebothrium* is reported to have a unique number of testes per proglottid and loculi per bothridia, these characters have been extremely plastic in the course of evolution and are of limited use, by themselves, in phylogenetic analysis. However, we would note that the members of *Rhinebothrium* having constricted bothridia, plus *R. lintoni* and *R. setiensis* which do not, comprise 13 of the 15 known species exhibiting 40 or more loculi per bothridium. In addition, the group of "constricted" species having long bothridial pedicels comprises 10 of the 15 known species with fewer than 15 testes per proglottid.

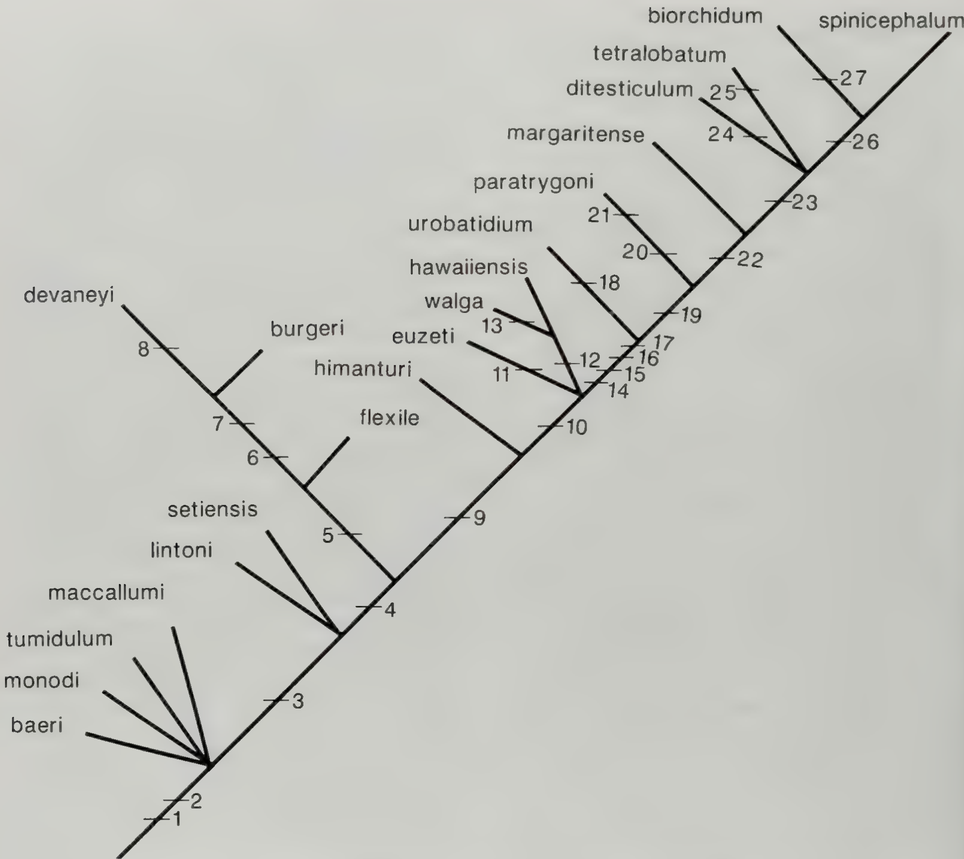


FIGURE 5. Cladogram depicting phylogenetic relationships of *Rhinebothrium* species having bothridial constrictions. Numbers accompanying slash marks on tree indicate synapomorphic traits as follows: (1) median septum in bothridia; (2) 17–22 testes per proglottid; (3) 42–56 loculi per bothridium; (4) constricted bothridia; (5) V-shaped ovaries; (6) muscular genital pore; (7) 30–43 testes per proglottid; (8) 96–154 loculi per bothridium; (9) bothridial pedicels ½ bothridial length; (10) 11–13 testes per proglottid; (11) 78 loculi per bothridium; (12) compact vitelline bands; (13) 4–6 testes per proglottid; (14) craspedote proglottids; (15) more than 50 proglottids per strobila; (16) square proglottids; (17) 6–12 ($\bar{x} = 8$) testes per proglottid; (18) 38–42 loculi per bothridium; (19) 4–8 ($\bar{x} = 5$) testes per proglottid; (20) long cephalic peduncle; (21) 72–76 loculi per bothridium; (22) 3–6 ($\bar{x} = 4$) testes per proglottid; (23) 2 ($\bar{x} = 2$) testes per proglottid; (24) long cephalic peduncle; (25) fragmented ovary; (26) 32–34 loculi per bothridium; (27) 22–30 loculi per bothridium. Not all characters for bottom 6 species listed.

It is evident from Figure 5 that while available data support a particular set of phylogenetic relationships, additional characters are needed to provide a stronger case and better resolution. Undoubtedly some postulated relationships will change as new data become available. However, the phylogenetic tree presented herein will provide a stable framework within which such changes can be made effectively. We may also evaluate the robustness of the tree presented in Figure 5 by reference to questions of evolutionary history and biogeography.

Brooks et al. (1981b) discussed the evolutionary history of the parasites inhabiting neotropical freshwater stingrays (family Potamotrygonidae). Their study suggested that the potamotrygonids were derived from a Pacific ancestor over 100 million years ago. One prediction of this hypothesis is that other neotropical species with marine affinities should have Pacific marine rel-

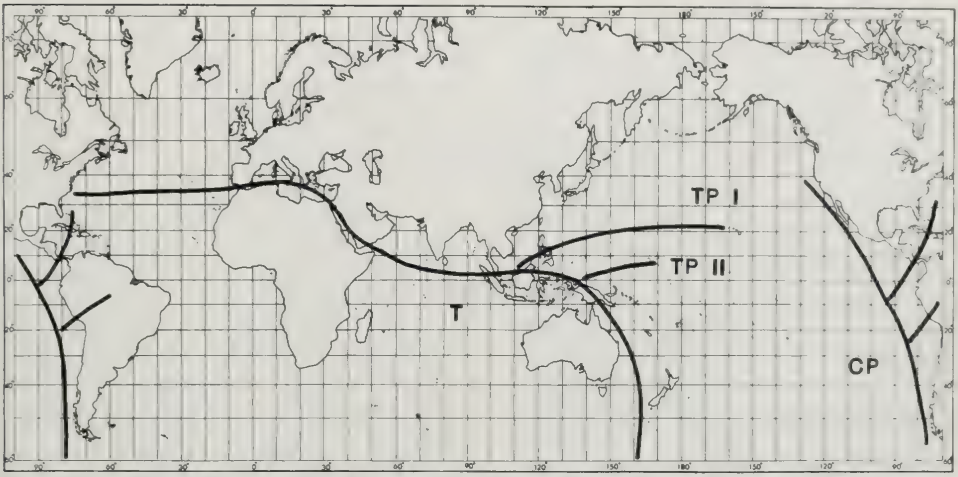


FIGURE 6. Biogeographic pattern of *Rhinebothrium* species having bothridial constrictions. Tethyan (=T) (Atlantic–Mediterranean–Indo-Malaysian): *R. baeri, R. monodi, R. tumidulum, R. maccallumi, R. lintoni, R. setiensis, R. flexile, R. burgeri, R. euzeti, R. walga.* Trans-Pacific I (=TP I) (Tethys–Hawaii): *R. hawaiiensis.* Trans-Pacific II (=TP II) (Tethys–Enewetak): *R. devaneyi.* Circum-Pacific (=CP): *R. himanturi* (Australia), *R. urobatidium, R. ditesticulum* (eastern Pacific); *R. paratrygoni* (South American freshwater); *R. margaritense, R. tetralobatum, R. biorchidum, R. spinicephalum* (Caribbean and west Atlantic).

atives. Nelson (1984) has shown a similar pattern for neotropical freshwater anchovies and their marine relatives. Another prediction presented by Brooks et al. (1981b) is that the relatively more plesiomorphic species in the parasite groups having members inhabiting potamotrygonids should have *circum-Pacific* distributions, indicating a biogeographic history stretching back to before the breakup of Gondwana. Furthermore, any members of such parasite groups that have *trans-Pacific* distributions should not be closely related to the species occurring in potamotrygonids.

Figure 6 shows the biogeographic pattern for *Rhinebothrium* species having bothridial constrictions. This is clearly a Pacific group, with only 3 Atlantic endemics, 2 of which occur in the Caribbean Sea. The species occurring in potamotrygonids (*R. paratrygoni*) is most closely related to circum-Pacific species, whereas *R. hawaiiensis* and *R. devaneyi,* the 2 trans-Pacific species, are most closely related to Indo-Malayan species. Thus, the biogeographic affinities of the 20 species of *Rhinebothrium* discussed herein support one of the predictions made by Brooks et al. (1981b). We stated above that the robustness of phylogenetic trees is increased by increasing the number of characters used. Biogeographic analyses are made more robust by demonstrating that more than one group of organisms show the same historical pattern of geographical distribution. We will now consider some of the members of the nematode genus *Echinocephalus,* one of which inhabits *U. asperrimus* from Enewetak.

Echinocephalus overstreeti Deardorff and Ko, 1983

Deardorff and Ko (1983) described the gnathostome nematode *Echinocephalus overstreeti* in *Taeniura melanopilos* from the Marquesas Islands. In addition to a new report of *E. overstreeti* in *U. asperrimus,* our findings also extend the geographic range of the species. Specimens of *E. overstreeti* in *U. asperrimus* from Enewetak are deposited in the BPBM as No. 1981.334.

Deardorff et al. (1980) presented a preliminary cladogram for part of *Echinocephalus* in conjunction with the description of *E. daileyi* from freshwater stingrays. Subsequently, Deardorff and Ko (1983) described *E. overstreeti* and discussed possible modifications in the cladogram presented by Deardorff et al. (1980). More recently, Beveridge (1985) redescribed *E. uncinatus* and *E. spinosissimus* and corrected previous reports of some morphological characters. Based on these subsequent studies and the specimens reported herein, we present an updated cladogram (Fig.

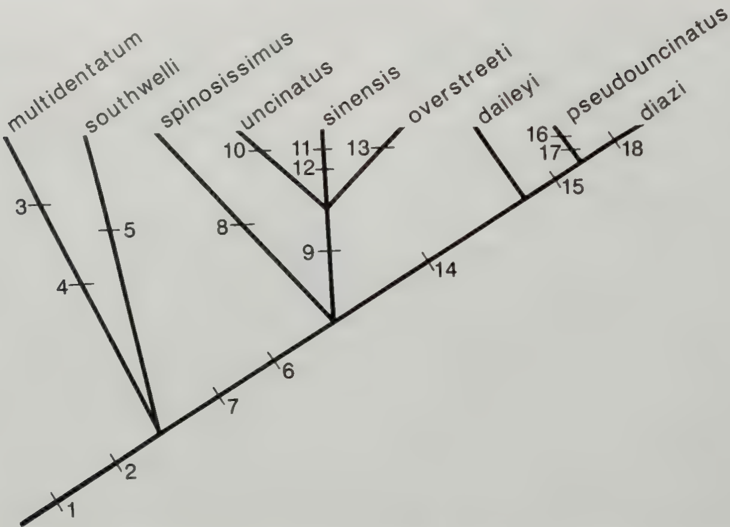


FIGURE 7. Cladogram, modified from Deardorff et al. (1980), depicting phylogenetic relationships of *Echinocephalus* species. Numbers accompanying slash marks on tree indicate synapomorphic traits as follows: (1) 5 pairs of postanal papillae; (2) 3 pairs of preanal papillae; (3) 15–18 rows of cephalic spines; (4) 4 pairs of postanal papillae; (5) 11–13 rows of cephalic spines; (6) 30–45 rows of cephalic spines; (7) gubernaculum present; (8) 5 pairs of postanal papillae in cluster; (9) ventral rugose areas surrounding male cloaca; (10) 5 pairs of postanal papillae in cluster; (11) 2 pairs of preanal papillae; (12) 26–29 rows of cephalic spines; (13) 3 pairs of postanal and 1 pair of adanal papillae; (14) 6 pairs of postanal papillae; (15) 25–27 rows of cephalic spines; (16) 3 pairs of postanal papillae; (17) 16–21 rows of cephalic spines; (18) 2 pairs of preanal papillae.

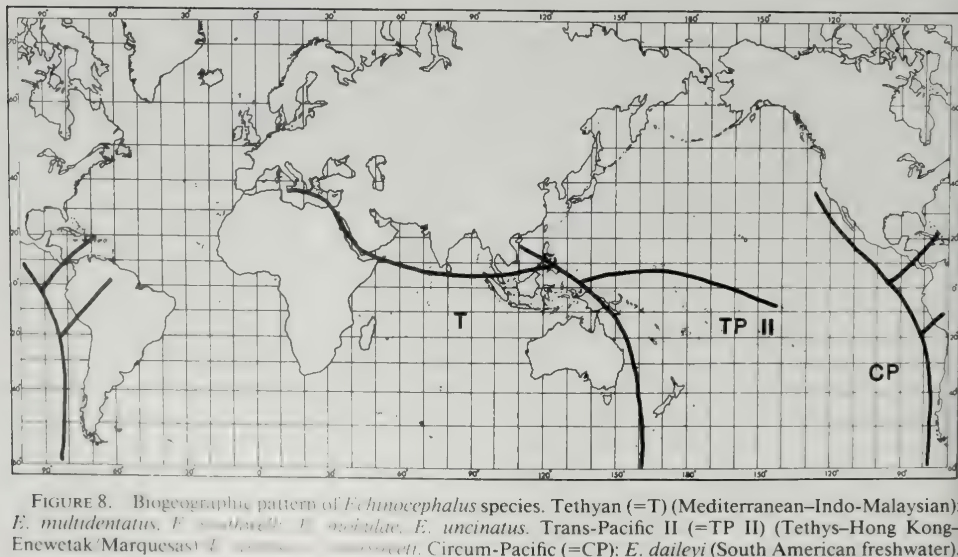


FIGURE 8. Biogeographic pattern of *Echinocephalus* species. Tethyan (=T) (Mediterranean–Indo-Malaysian): *E. multidentatus*, *E. southwelli*, [illegible], *E. uncinatus*. Trans-Pacific II (=TP II) (Tethys–Hong Kong–Enewetak/Marquesas): [illegible]. Circum-Pacific (=CP): *E. daileyi* (South American freshwater), *E. pseuduncinatus* (Eastern Pacific), *E. diazi* (Caribbean).

7) based on a suite of 18 anatomical characters listed in the figure legend. The present phylogenetic hypothesis differs from that of Deardorff et al. (1980) in 3 ways. First, *E. overstreeti* has been included and is postulated to be a sister-species of *E. sinensis* (see also Deardorff and Ko, 1983). Second, *E. spinosissimus* is included as a valid species. And third, both *E. uncinatus* and *E. spinosissimus*, by virtue of possessing a gubernaculum in males, belong in a clade containing all species except *E. multidentatum* and *E. southwelli*. The relative relationships of the *E. sinensis* group and the *E. daileyi–E. pseudouncinatus–E. diazi* clade remain the same as postulated by Deardorff et al. (1980).

Phylogenetic trees can be used to predict previously undetected biological similarities in closely related species (Brooks, 1985). The larval stages of *E. sinensis* Ko, 1975, have been shown to be capable of penetrating the gastrointestinal tract and undergoing random visceral migrations in a variety of mammals (Ko et al., 1975; Ko, 1976). This species, therefore, may represent a human health risk to consumers of the raw oyster, *Crassostrea gigas*, in Hong Kong, Southern China, where the worm's life cycle is known to exist. To date, no other species of *Echinocephalus* has been tested for invasiveness. However, as Figure 7 shows, *E. sinensis* is most closely related to *E. overstreeti*. Perhaps the invasive potential of *E. overstreeti* is also similar to that of *E. sinensis*. The third-stage larvae of *E. overstreeti* are found in an edible mollusc (unpubl. obs.).

Figure 8 shows the biogeographic pattern indicated by that cladogram. Note that *E. overstreeti*, a trans-Pacific species, is most closely related to *E. daileyi*, the species found in potamotrygonids. Thus, for 2 parasite groups investigated so far, the predictions by Brooks et al. (1981b) have been corroborated. This strengthens the hypothesis of ancient Pacific origins for potamotrygonid stingrays.

ACKNOWLEDGMENTS

We acknowledge the late Dr. Dennis M. Devaney, at the BPBM, Honolulu, for loaning of specimens and Dr. John E. Randall, also at the BPBM, for providing the host identification. This study was partially funded by operating grant A7696 from the Natural Sciences and Engineering Research Council of Canada to D.R.B.

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J. Parasit., 74(3), 1988, pp. 466–470

THE GENUS *MYRMOZERCON* BERLESE, WITH DESCRIPTIONS OF TWO NEW SPECIES (ACARI: MESOSTIGMATA: LAELAPIDAE)

Rose Marie T. Rosario and Preston E. Hunter
Department of Entomology, University of Georgia, Athens, Georgia 30602

ABSTRACT: The genus *Myrmonyssus* Berlese, 1903, is synonymized with *Myrmozercon* Berlese, 1902. Two new species are described and illustrated. Palpal and leg chaetotaxy for certain species are given.

In 1902 Berlese erected the genus *Myrmozercon* (type species, *M. brevipes*), and the following year (Berlese, 1903a) erected the genus *Myrmonyssus* (type species, *M. diplogenius*) for new species of mites collected from ants. Illustrations were not included with the original descriptions, but both genera and their type species were redescribed and the species illustrated in a separate paper (Berlese, 1903b). Three additional species of *Myrmonyssus* were also described and illustrated in the later paper.

Myrmozercon presently includes 2 species and *Myrmonyssus* 12 species (1 of questionable status). All species in both genera have been collected from or in association with ants. The 2 genera share common characters, and each genus, according to descriptors, has "unique" characters. A comparison of species of *Myrmozercon* and *Myrmonyssus* based on descriptions, illustrations, and specimens available to us showed that the "unique" characters ascribed to each genus are not confined to that genus, and that differences between the genera are often a matter of the degree of development rather than presence or absence of characters (see Remarks). On the basis of this information, we consider *Myrmonyssus* Berlese, 1903, to be a synonym of *Myrmozercon* Berlese, 1902. In this paper we redefine *Myrmozercon*, describe 2 new species, and provide information on palpal and leg chaetotaxy for certain species.

REDESCRIPTION

Myrmozercon Berlese, 1902

Myrmozercon Berlese, 1902, Zoologischer Anzeiger **25**(683–684): 699.
Myrmonyssus Berlese, 1903, Zoologischer Anzeiger **27**: 16 (new synonymy).
Myrmozercon: Berlese, 1903, Redia **1**: 444–445.
Myrmonyssus: Berlese, 1903, Redia **1**: 436–437.

Diagnosis: Dorsal shield entire, blunt on posterior margin, covering dorsum except small area at posterior of idiosoma; shield may or may not be densely covered with setae. Anal shield separate, usually terminal in position; shield may be enlarged with 1 pair of ventral setae and 3 anal setae. Peritreme usually not extending anterior to coxae II. All tarsi with well-developed ambulacrum, without claws. Palpal trochanter with 1 seta, seta v2 absent. Chelicerae never strongly toothed, normally edentate, fixed digit reduced or absent. Female sternal shield fused to well-developed endopodal shields, or sternal shield more or less truncated posteriorly and weakly fused to narrow endopodal shields; genito-ventral shield wide, narrowing posteriorly, ending anterior to anal shield. Male with sterno-genito-ventral shield, ending posteriorly as in female, separate from anal shield.

Type species: Myrmozercon brevipes Berlese, 1902; collected with ants in Umbria, Italy (by original designation).

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DESCRIPTIONS

Myrmozercon robustisetae n. sp.

Male: Dorsum (Fig. 1C). Dorsal shield 404 μm long, 325 μm wide (average of 2 specimens), covering entire idiosoma except small opisthonotal area, posterior margin slightly concave; shield densely covered with long, simple setae; surface of shield reticulate. Integument posterior to shield bearing 1 pair of setae. *Ventrum* (Fig. 1D). Sterno-genito-ventral shield fused with endopodals, extending between coxal bases, with small extension posterior to coxae IV, bearing 8 pairs of simple setae, posterior pair longest, reaching bases of paranal setae; anterior margin of shield straight, posterior margin forming rounded point, almost touching anal shield. Metapodal shields small. Exopodal shield thin. Anal shield triangular, longer than wide, on posterior margin of ventrum, anterior margin with slight incision on middle, bearing 1 pair long, thick opisthonotal setae; paranals and anal setae thick, spinelike, all located posterior to anal opening. Peritremes long, extending beyond margin of coxae I; peritremal shield extending anteriorly beyond peritreme and posteriorly behind level of coxae IV. Small platelet present between peritremal shield and extension of sterno-genito-ventral shield. Opisthonotal integument bearing 2 pairs of simple setae, shorter than other ventral setae, integument well striated. *Gnathosoma* (Fig. 1B). Setae simple, relative lengths and thickness as illustrated; deutosternal groove bearing 8 rows of small denticles; anterior margins of hypostome rounded; corniculi small, weakly sclerotized. Number of setae on palpal trochanter, femur, and genu as follows: 1, 4, 4. Chelicerae edentate, movable digit small; anterior portion of fixed digit hyaline-

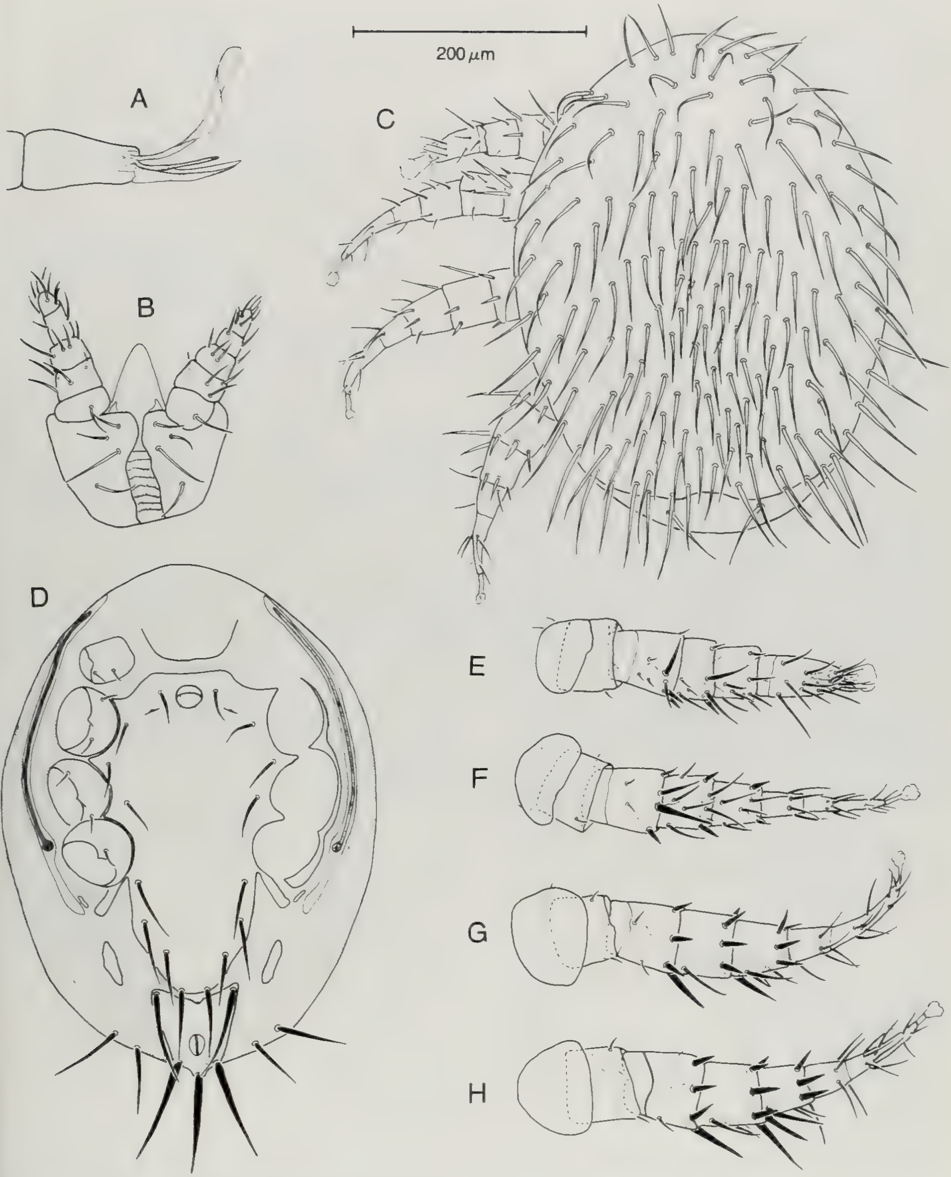

FIGURE 1. *Myrmozercon robustisetae* n. sp., male. (**A**) chelicera; (**B**) ventral view of gnathosoma; (**C**) dorsum; (**D**) ventrum; dorsal view of leg I (**E**), leg II (**F**), leg III (**G**), and leg IV (**H**).

like, blunt, scooplike; spermatodactyl enclosing base of movable digit (Fig. 1A). *Legs.* Some dorsal leg setae thick, spinelike (Fig. 1E–H). Leg chaetotaxy given in Table I.

Female: Unknown.

Type specimens

Host and locality: Holotype and 1 paratype, both males, from termite's nest, Puerto Rico, 8 April 1968, collected by R. Wiegert.

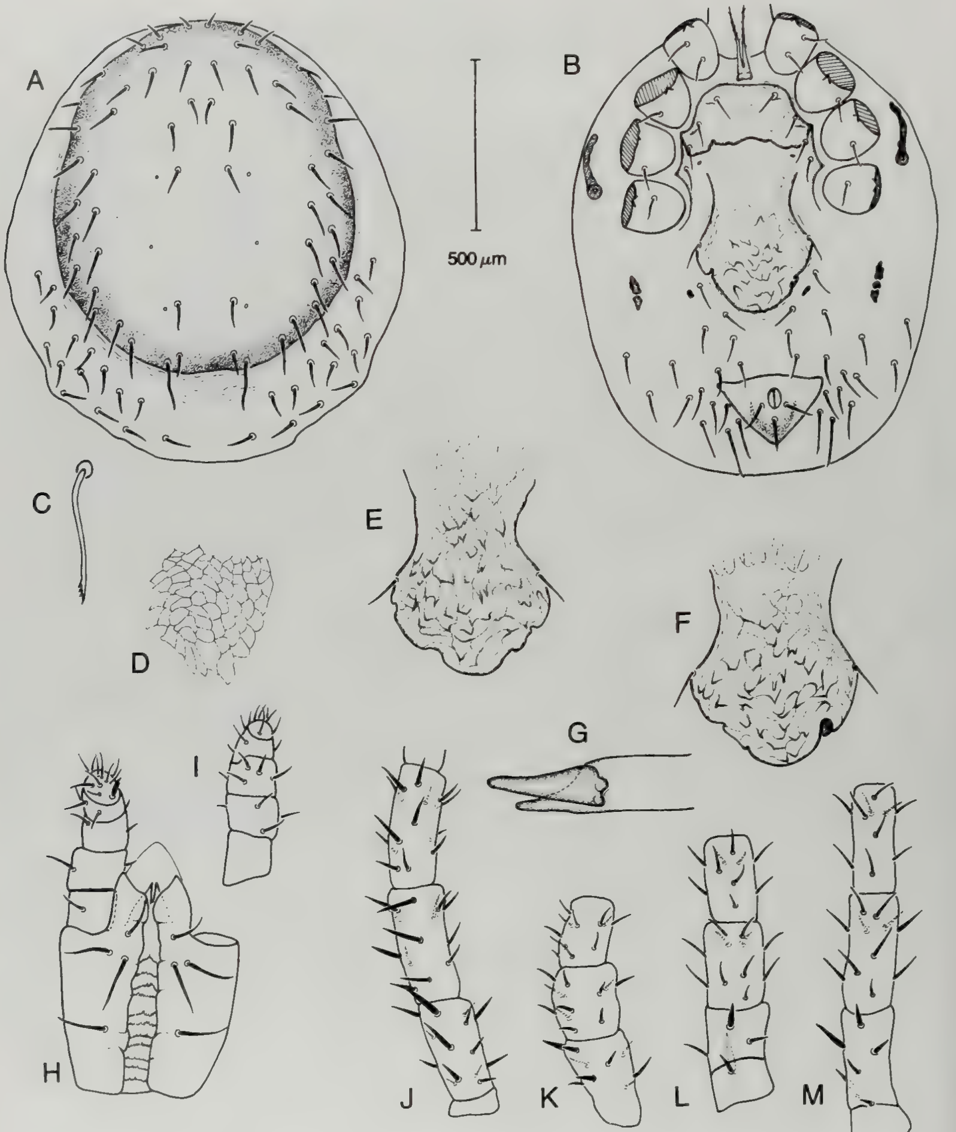

FIGURE 2. *Myrmozercon rotundiscutum* n. sp., female. (**A**) dorsum; (**B**) ventrum; (**C**) seta Z5; (**D**) dorsal shield reticulations; (**E, F**) genital shields of paratypes; (**G**) chelicera; (**H**) ventral view of gnathosoma; (**I**) dorsal view of palp; dorsal view of femur, genu, and tibia of leg I (**J**), leg II (**K**), leg III (**L**), and leg IV (**M**).

Holotype: Deposited in the National Museum of Natural History, Washington, D.C.

Paratype: Deposited in the Acarology Collection, Department of Entomology, University of Georgia, Athens, Georgia 30602.

Diagnosis

This species can be recognized by the following combination of characters: dorsal shield densely covered with long, simple setae; anal and paranal setae long,

TABLE I. *Leg chaetotaxy (coxa to tibia) of* Myrmozercon *species.*

Species	Leg I	Leg II	Leg III	Leg IV
clarus	2-5-8-8-8	2-5-8-8-7	2-4-6-8-7	1-4-7-8-7
spinosus	2-5-8-8-8	2-4-8-8-7	2-4-6-8-7	1-4-6-7-7
rotundiscutum	2-6-14-13-13	2-5-9-11-11	2-5-5-11-10	1-5-6-11-10
robustisetae	2-5-10-9-9	2-5-8-10-8	2-5-7-9-8	1-5-7-9-8

thick; anal shield bearing 1 pair of ventral setae and 3 anal setae; some dorsal leg setae spinelike.

Etymology

The species name *robustisetae* comes from the Latin *robustus* and *seta* referring to the thick anal setae.

Comments

Because of the dorsal setation and the presence of 5 setae on the anal shield, the female of *M. robustisetae* should be easily recognized. The collection of this species from a termite nest may be misleading as ants are often in the vicinity of such nests.

Myrmozercon rotundiscutum n. sp.

Female: Dorsum (Fig. 2A). Shield 916 μm long, 772 μm wide (average of 3 specimens), not covering idiosoma, ending short of posterior margin of dorsum; shield bearing 27 pairs of setae, setae simple except Z5, which are weakly setose at tip (Fig. 2C); setae Z5 longer than other dorsal setae; surface of shield with striation (Fig. 2D); integument around opisthonotal area of shield bearing 15 pairs of simple setae. *Ventrum* (Fig. 2B). Sternal shield well sclerotized, bearing 3 pairs of simple setae; anterior margin of shield rounded, without anterolateral point extending between coxae I and II, posterior margin irregular; shield surface striated; shield weakly fused to narrow endopodal shields. Metasternal shields absent, setae arising from integument. Genito-ventral shield well sclerotized, bearing genital setae only; shield surface striated; posterior margin rounded, irregular in shape (Fig. 2E, F), ending short of anal shield. Metapodal shield consisting of 2–3 platelets. Exopodal shield represented by thin line at most. Anal shield triangular, wider than long; paranal setae arising at posterior level of anal opening, setae length approximately equal to postanal seta; postanal seta thicker than paranals (anal shield located at posterior margin of body in paratypes; due to mounting holotype shows shield submarginal). Peritremes short, not extending to anterior margin of coxae II; peritremal shield weakly developed. Opisthonotal integument bearing 16 pairs of setae, all setae simple except 2 pairs behind anal shield, which bear 2–3 small spines near their tips; relative lengths of setae as illustrated. *Gnathosoma* (Fig. 2H, I). Setae simple, relative lengths and thickness as illustrated; deutosternal groove bearing 11 rows of small denticles; anterior margins of hypostome lobelike, extending anterior to palpal trochanter and covering base of corniculi; corniculi weakly sclerotized. Number of setae on palpal trochanter femur and genu as follows: 1, 4, 5. Chelicerae chelate, edentate (Fig. 2G). *Legs.* Tarsi without claws, each with well-developed ambulacrum; some dorsal leg setae spinelike (Fig. 2J–M), all other setae simple. Leg chaetotaxy, following Evans (1963), given in Table I.

Male: Unknown.

Type specimens

Host and locality: Holotype and 2 paratypes, all females, from *Camponotus* sp. (ant), Idaho: Elmore Co.: Dismal Swamp, T5N, RgE, 934, 2,135 m elev., collected by Mike Twitchell. Although not stated in collection data, it is believed that the material was collected in 1985.

Holotype: Deposited in the National Museum of Natural History, Washington, D.C.

Paratype: One deposited in the collection of Dr. Asher E. Treat, P.O. Box 51, Tyringham, Massachusetts 02164; one paratype deposited in the Acarology Collection, Department of Entomology, University of Georgia, Athens, Georgia 30602.

Diagnosis

Myrmozercon rotundiscutum is distinct in having the genito-ventral shield rounded posteriorly and ending well short of the anal shield, narrow endopodal shields, and on the dorsal shield setae Z5 are longer than other dorsal setae.

Etymology

The species name *rotundiscutum* comes from the Latin *rotundus* and *scutum* referring to the rounded genito-ventral shield of the female.

REMARKS

Specimens of *Myrmozercon clarus* (Hunter and Hunter, 1963) and *M. spinosus* (Hunter and Hunter, 1963) and the 2 new species described above were available to us for comparisons.

Neoteny of the palp trochanter is exhibited by these species and in illustrations of other species. The free-living adult pattern for palpal trochanter, femur, and genu is 2-5-6 (Evans, 1964). The setal complements for the same segments of specimens available to us are as follows: *spinosus* and *clarus*: 1-5-6; *rotundiscutum* n. sp.: 1-4-5; and *robustisetae* n. sp.: 1-4-4.

Variation also occurs in the development of the metasternal–endopodal, genito-ventral, and anal shields, and dorsal sectation in *Myrmozercon* species. Berlese's (1903b) illustration of new species he placed in *Myrmonyssus* (*diplogenius,*

acuminatus, and *antennophoroides*) showed narrow endopodal shields as in *rotundiscutum* n. sp. Other species described in *Myrmonyssus* (*clarus*; *spinosus*; *eidmanni* Sellnick, 1941; *liguricus* Vitzthum, 1930) have the sternal, metasternal, and endopodal shields fused into a horseshoe shape as illustrated by Berlese (1903b) for *Myrmozercon brevipes*. The anal shield may be longer than wide (*brachiatus* Berlese, *acuminatus* Berlese, *liguricus*), wider than long (*eidmanni*, *clarus*, *spinosus*, *rotundiscutum* n. sp.), or enlarged and bearing 1 pair of opisthonotal setae (*chapmani* Baker and Strandtmann, 1948; *robustisetae* n. sp.). The dorsal shield bearing dense, short setae was given as a generic character of *Myrmozercon*. However, this condition also occurs in *Myrmozercon scutellatus* (Hull, 1923).

ACKNOWLEDGMENT

We thank Dr. Asher Treat for the loan of his specimen of *rotundiscutum*.

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SCHISTOSOMA MALAYENSIS N. SP.: A *SCHISTOSOMA JAPONICUM*-COMPLEX SCHISTOSOME FROM PENINSULAR MALAYSIA

George J. Greer*, C. K. Ow-Yang, and Hoi-Sen Yong†
Division of Parasitology, Institute for Medical Research, Kuala Lumpur, Malaysia

ABSTRACT: *Schistosoma malayensis* n. sp., a member of the *Schistosoma japonicum* complex is described from *Rattus muelleri* in Peninsular Malaysia and 2 strains are characterized. The only morphological differences noted among adults from natural hosts were that *S. malayensis* are in general smaller than *S. mekongi* and *S. japonicum*. But these differences may be the result of host-induced variations and therefore are of little taxonomic value. To minimize the effects of host-induced variations, adult worms recovered from laboratory mice with similar worm burdens at 50–56 days postinfection were compared. These comparisons revealed only minor morphometric differences among these 3 species. *Schistosoma malayensis* eggs from naturally and experimentally infected hosts are most similar to those of *S. mekongi*, with eggs of both species being, in general, smaller than those of *S. japonicum*. The egg index for *S. malayensis* is usually higher than for *S. japonicum* and lower than for *S. mekongi*. Differences were noted in the developmental rates in mice for 2 isolates of *S. malayensis*, *S. mekongi*, and *S. japonicum* (Philippine strain), but relatively large differences observed between isolates of *S. malayensis* indicate that, in this case, the developmental rate is not a useful taxonomic character. *Schistosoma malayensis* is erected principally on the basis of differences, reported elsewhere, in the life histories and in the electrophoretic migration patterns of isoenzymes of adult worms as compared to *S. mekongi* and *S. japonicum*. These comparisons indicate that *S. malayensis* is more closely related to *S. mekongi* than to *S. japonicum*.

Schistosoma japonicum-like eggs have been recovered from tissues of 10 aborigines (Orang Asli) in Peninsular Malaysia (Murugasu and Dissanaike, 1973; Leong et al., 1975; Murugasu et al., 1978). In 1978, aquatic snails, *Robertsiella kaporensis*, and wild rats, *Rattus muelleri*, infected with this parasite were collected at the Sungai Kapor, a small stream in the foothills of the main mountain range of Peninsular Malaysia (Greer et al., 1980). During extensive surveys for snail hosts of this schistosome, Greer et al. (1984a) discovered 2 additional sites of transmission in Peninsular Malaysia where 2 other species of *Robertsiella* serve as intermediate hosts. Studies were undertaken to determine the taxonomic status of the schistosomes transmitted by these snails.

Schistosoma mekongi Voge, Buckner, and Bruce, 1978, was described as a new species based principally on biological differences between that parasite and strains of *S. japonicum*. Those authors used infections of laboratory mice to compare morphological and biological characteristics. For our studies we have employed a similar approach and have in addition compared worms and eggs obtained from naturally infected hosts. Based on these comparisons and differences in the life histories and electrophoretic isoenzyme patterns of *S. japonicum*-complex schistosomes, reported elsewhere, we describe the Malaysian schistosome as a new species and characterize 2 strains of this parasite.

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* Present address: Department of Tropical Medicine, Tulane University Medical Center, 1430 Tulane Ave., New Orleans, Louisiana 71102.

† Jabatan Genetik dan Biologi Sel, Universiti Malaya, Kuala Lumpur, Malaysia.

MATERIALS AND METHODS

Animals

Schistosoma malayensis was obtained from naturally infected snail and mammalian hosts collected at the Sungai Kapor (Sungai means stream or river in Malay) (4°15′50″N, 101°51′45″E) near Kuala Koyan, Pahang State; at the Sungai Wa (4°25′15″N, 102°24′00″E) near Kuala Tahan, Pahang; and at a tributary of the Sungai Charok Bukit Sebelah (5°42′30″N, 100°58′15″E) about 10 km from Baling, Kedah (Fig. 1). Naturally infected rats were kept in the laboratory for at least 1 mo before sacrifice. Cercariae used to infect mice were obtained from both natural and experimental infections.

Schistosoma japonicum was obtained from natural and laboratory infected snails collected from Palo, Leyte, the Philippines and was graciously provided by Dr. Edito Garcia, Institute of Public Health and Dr. John Cross, NAMRU 2, Manila. *Schistosoma mekongi*, originally isolated from Khong Island, Laos, in 1970, was graciously provided by Drs. Santasiri Sornmani and Viroj Kitikoon, Mahidol University, Bangkok. ICR female mice, 20–25 g, obtained from the Division of Laboratory Animal Resources, Institute for Medical Research, were used for all experimental infections.

FIGURE 1. Map of Peninsular Malaysia showing sites where *Schistosoma malayensis*-infected snails and rats were collected. **A.** Kuala Tahan, Pahang. **B.** Kuala Koyan, Pahang. **C.** Baling, Kedah.

Procedures

Mice were infected by loop method with 20–30 cercariae and subsequently maintained at ambient temperatures, range 24–37 C. Worms were recovered by dissection from mice killed by cervical dislocation between 20 and 70 days postinfection. Worms from mice with 8 or fewer pairs were used for morphometric studies. *Schistosoma malayensis* was recovered by dissection from *Rattus muelleri* and *R. tiomanicus* killed by pentobarbital overdose.

Worms were transferred to RPMI-1640 culture medium (GIBCO) with 10% fetal calf serum, briefly washed in PBS (pH 7.2), fixed in Carnoy's fixative, stained with Semichon's carmine, and mounted in Permount.

Measurements used in the adult worm descriptions and for other morphometric comparisons presented in this report are based on measurements from at least 20 male and 20 female worms, with the exception of *R. tiomanicus* captured at Baling and *R. muelleri* from Kuala Tahan, which are based on 5 and 6 worm pairs, respectively. Measurements for tests are based on at least 4 testes from each male worm.

Liver squashes from mice were checked for eggs by microscopic examination beginning on day 25. Egg measurements from mice are based on a minimum of 100 eggs with fully developed miracidia recovered from livers of mice sacrificed between 50 and 52 days postinfection. For each isolate, results are based on eggs recovered from 4 or more mice. Egg size from natural infections are based on measurements of at least 100 eggs recovered from the feces of each host species. The egg index was calculated as the ratio of width/length × 100 (Hsu and Hsu, 1958).

Schistosoma malayensis cercariae from laboratory and naturally infected snails were used for morphological studies. Cercariae, collected from pooled infected snails, were killed by gently heating over an alcohol flame and were measured under light coverslip pressure. Measurements are based on 20 cercariae.

All measurements are in microns unless indicated otherwise.

RESULTS

Schistosoma malayensis n. sp.

(Figs. 2–4)

Description

Male (23 worms Koyan strain): Body spined, 4.30–9.21 (6.82) mm long by 0.24–0.43 (0.32) mm wide. Oral sucker terminal, 153–264 (212) long by 164–279 (214) wide. Ventral sucker 208–335 (269) long by 195–353 (274) wide, anterior edge located 186–409 (308) from posterior margin of oral sucker. Esophagus 193–365 (273) long, terminates near ventral sucker where it is surrounded by a cluster of gland cells. Ceca unite in posterior half of body. Gynecophoral canal extends from just posterior to ventral sucker to posterior end of body. Testes, 6–8 in number; 51–157 (95) long by 99–230 (147) wide; oval; contiguous; in series beginning near anterior end of gynecophoral canal. Seminal vesicle immediately to slightly anterior to first testis, discharges through duct at pore near anterior end of gynecophoral canal.

Female (23 worms Koyan strain): Body spined, 6.48–11.28 (9.16) mm long by 0.15–0.28 (0.21) mm wide. Oral sucker terminal, 46–99 (72) long by 27–61 (49) wide. Ventral sucker 13–23 (18) long by 16–28 (22) wide, anterior edge located 97–219 (143) from posterior margin of oral sucker. Esophagus 83–169 (123) long terminates near ventral sucker. Ceca unite immediately posterior to ovary; cecum terminates near posterior end of body. Ovary 365–763 (533) long by 99–141 (120) wide; elongate; smooth or convoluted; anterior margin in anterior half of body; 1.83–5.12 mm from anterior end. Seminal receptacle not present. Ootype anterior to ovary. Uterus contains numerous eggs; intercecal; extends from ootype to genital pore which opens immediately posterior to ventral sucker. Vitellaria occupy posterior two-thirds to one-half of body.

Egg: Oval; 52–90 (67) long by 33–62 (54) wide; small knob usually located laterally, occasionally near end of egg.

Cercaria: General description same as for *S. japonicum* (Cort, 1921). Body 127–176 (154) long by 46–66 (55) wide. Head organ 44–69 (58) long by 35–44 (39) wide. Tail stem 118–189 (151) long by 18–40 (27) wide, furcae 59–97 (71) long.

Holotype: U.S. National Museum Helminth Collection No. 78118, a female (Koyan strain).

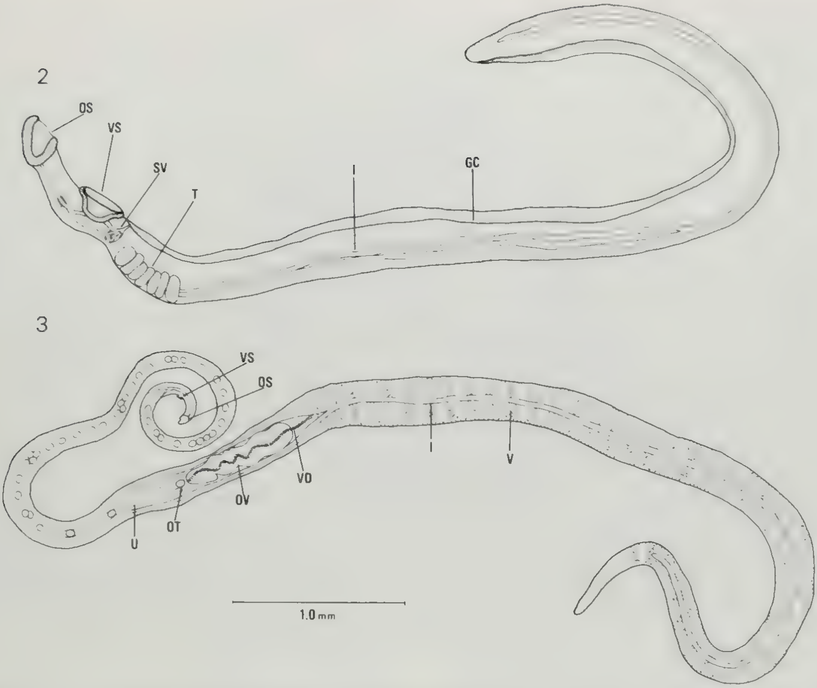
Allotype: USNM Helm. Coll. No. 78119, a male (Koyan strain).

Paratypes: USNM Helm. Coll. No. 78120, females and males (Koyan strain); and Nos. 78121 and 78122, females and males (Baling strain).

Type host: Rattus muelleri (Jentink), American Museum of Natural History No. 246094.

Other mammal hosts: Homo sapiens (L); *R. tiomanicus jalorensis* (Miller), AMNH No. 232454.

Site of infection: Mesenteric and hepatic portal veins.



FIGURES 2, 3. *Schistosoma malayensis* from *Rattus muelleri*. **2.** Female. **3.** Male. GC = gynecophoral canal; I = intestinal cecum; OS = oral sucker; OT = ootype; OV = ovary; SV = seminal vesicle; T = testes; U = uterus; V = vitellaria; VD = vitelline duct; VS = ventral sucker.

Intermediate host at type locality: Robertsiella kaporensis Davis and Greer, 1980, AMNH No. 213822.

Other intermediate hosts: Robertsiella gismanni Davis and Greer, 1980, AMNH NO. 213833; an undescribed species of *Robertsiella*, AMNH No. 213824.

Type locality: Kuala Koyan, Pahang State, Malaysia.

Etymology: Specific epithet refers to the country where this species was discovered.

Comparisons of *S. malayensis* isolates

Schistosoma malayensis was recovered from 3 sites in Peninsular Malaysia (Fig. 1). The snail and mammalian hosts, respectively, for each site are as follows: *Robertsiella kaporensis–Rattus muelleri*, Kuala Koyan, Pahang; *R. gismanni–R. muelleri*, Kuala Tahan, Pahang; and an undescribed species of *Robertsiella–R. muelleri* and *R. tiomanicus*, near Baling, Kedah.

Tables I and II show measurements for male and female *S. malayensis*, respectively, from *R. muelleri* captured at the Kuala Koyan and Baling sites. The Koyan isolate is larger overall, but ranges overlap for all measurements except for the distance between suckers and for the esophageal length of females.

Measurements of a small number of worms from *R. muelleri*–Kuala Tahan were found to be most like those of worms from *R. muelleri*–Kuala Koyan and worms from *R. tiomanicus*–Baling were most like those from *R. muelleri*–Baling.

Differences in developmental rates of these isolates in mice were noted. Eggs were first seen in liver tissue of mice at 30 days postinfection for the Baling isolate and at 33 days postinfection for the Koyan isolate. Eggs containing fully developed miracidia were first present at 38 and 43 days, respectively.

Comparisons of *S. malayensis*, *S. mekongi*, and *S. japonicum*

Morphometric comparisons of these schistosomes from mice 50–56 days postinfection are shown in Tables III and IV. Except for the testis and ovary measurements, *S. malayensis* (B) is in general smaller than other isolates. However, ranges overlap for all measurements in these tables.

Table V compares results from several sources for the average egg size and egg index of *S. malayensis*, *S. mekongi*, and *S. japonicum* eggs recovered from the feces of natural hosts. *Schistosoma mekongi* eggs from man and dog are smaller and more rounded in shape, i.e., have a higher index, than those of *S. japonicum* from the same hosts. *Schistosoma malayensis* eggs from rats are roughly the same size as those of *S. mekongi* from man and dog. In general the egg indices for *S. malayensis* isolates fall between those of *S. mekongi* and *S. japonicum* with the exception of *S. malayensis* eggs from *R. muelleri*–Baling, which have a lower egg index than *S. japonicum* (J) from dogs.

Table VI compares the results from several sources

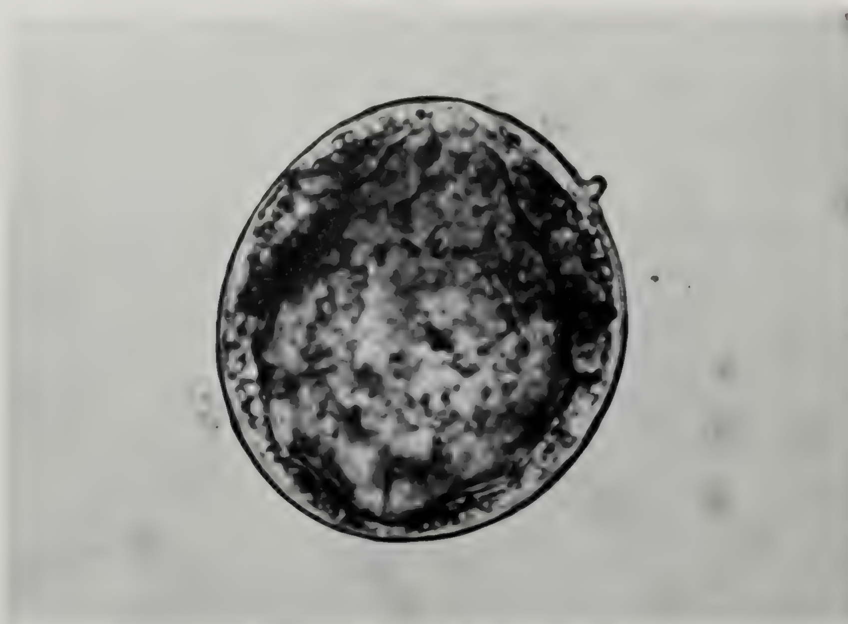


FIGURE 4. *Schistosoma malayensis* egg from the feces of *Rattus muelleri.*

for the average egg size and egg index for *S. malayensis, S. mekongi,* and *S. japonicum* recovered from experimentally infected laboratory mice. Measurements for eggs reported by Voge et al. (1978), based on eggs in liver tissue at 35 days, are considerably smaller than those of eggs from mouse stool (Hsu and Hsu, 1958) and from mouse liver at 50–52 days (present study). Egg indices from Voge et al. for *S. japonicum* are in general similar to other reports, but the index for *S. mekongi* is much lower than that found in our study. These discrepancies may be the result of different periods postinfection chosen for examination of eggs.

Our results presented in Tables V and VI show that, although egg indices are comparable, eggs of *S. malayensis* and *S. mekongi* from mouse liver are considerably larger than eggs of the same species from the feces of natural hosts. We have no adequate explanation for these differences.

DISCUSSION

Our morphological observations revealed no noteworthy differences among *S. malayensis, S. mekongi,* and *S. japonicum* adult worms. It therefore appears that Voge et al. (1978) were mistaken when they stated in the description of *S. mekongi* that the ootype is located posterior to the ovary. In all 3 species the ootype is located just anterior to the ovary.

Morphometric comparisons of specimens from

TABLE I. *Comparative measurements of male* Schistosoma malayensis *recovered from* Rattus muelleri *captured near Kuala Koyan, Pahang, and Baling, Kedah, Malaysia.*

Strain	Body		Oral sucker		Ventral sucker		Distance between suckers	Esophagus length	Testis number	Testis	
	Length*	Width*	Length	Width	Length	Width				Length	Width
Koyan	6.82†	0.32	212	214	269	274	308	273	6–8	95	147
	4.30–9.21‡	0.24–0.43	153–264	164–279	208–335	195–353	186–409	193–365		51–157	99–230
	±1.39§	±0.05	±29	±29	±33	±33	±60	±46		±22	±29
Baling	4.31	0.23	145	144	194	185	234	213	6–8	69	122
	3.22–5.27	0.18–0.27	108–182	112–171	149–227	86–257	149–353	149–279		42–126	74–175
	[illegible]	±0.03	±21	±15	±23	±44	±51	±35		±18	±20

* In millimeters.
† Mean.
‡ Range.
§ Standard deviation

TABLE II. *Comparative measurements of female worms and eggs of* Schistosoma malayensis *recovered from* Rattus muelleri *captured near Kuala Koyan, Pahang, and Baling, Kedah, Malaysia.*

Strain	Body Length*	Body Width*	Oral sucker Length	Oral sucker Width	Ventral sucker Length	Ventral sucker Width	Distance between suckers	Esophagus length	Ovary Length	Ovary Width	Embryonated egg Length	Embryonated egg Width
Koyan	9.16†	0.21	72	49	18	22	143	123	533	120	67	54
	6.48–11.28‡	0.15–0.20	46–99	27–61	13–23	16–28	97–219	83–169	365–763	99–141	52–90	33–62
	±1.41§	±0.03	±12	±9	±4	±3	±33	±30	±107	±11	±6	±6
Baling	5.27	0.17	50	47	15	25	62	70	423	106	65	50
	3.66–6.74	0.12–0.22	37–71	37–60	9–21	21–30	46–80	58–79	236–681	81–121	57–81	40–58
	±0.81	±0.02	±10	±6	±3	±3	±12	±7	±95	±11	±5	±4

* In millimeters.
† Mean.
‡ Range.
§ Standard deviation.

Rattus muelleri indicate that *S. malayensis* adults from Baling, Kedah, are considerably smaller than those from Kuala Koyan, Pahang. Adults of both *S. malayensis* isolates are smaller in general than those of *S. japonicum* (Faust and Meleney, 1924) and *S. mekongi* (Iijima et al., 1971; Voge et al., 1978) recovered from natural hosts. However, morphometric comparisons of adult trematodes from natural hosts are of little value because variability may be associated with host species, age and health of the hosts, worm burdens, and other factors. *Schistosoma malayensis* eggs from natural rat hosts closely resemble those of *S. mekongi* from dog and man, differing in having a somewhat lower egg index. *Schistosoma japonicum* eggs from natural hosts are generally larger and have a lower egg index than either *S. malayensis* or *S. mekongi*.

In an attempt to minimize the effects of host-induced variations, worms of roughly the same age and from mice with similar worm burdens were used for extensive morphometric comparisons. Although comparisons among these 3 species revealed many differences in average measurements, in most cases ranges broadly overlap. Our conclusions are in agreement with Voge et al. (1978), who suggested that egg shape and size are the only morphological features useful for differentiation among these *S. japonicum*-complex schistosomes. Yet, even these features are of limited value for distinguishing between *S. malayensis* and *S. mekongi*.

Differences in developmental periods in experimentally infected mice have been used as a characteristic feature among strains and species of the *S. japonicum*-complex. Voge et al. (1978) followed development in mice to the appearance of the first fully developed eggs in the liver and found that *S. mekongi* lagged 7–8 days behind 4 strains of *S. japonicum*. We also observed a pro-

TABLE III. *Comparative measurements of male* Schistosoma malayensis *(Koyan and Baling strains),* S. mekongi, *and* S. japonicum *(Philippine strain) from mice 50–60 days postinfection.*

Schistosome (strain)	Oral sucker Length	Oral sucker Width	Ventral sucker Length	Ventral sucker Width	Distance between suckers	Testis number	Testis Length	Testis Width
S. malayensis (Koyan)	178*	190	246	233	293	6–7	71	119
	130–294†	150–242	171–316	140–301	223–390		39–123	67–182
	±37‡	±23	±38	±40	±44		±15	±27
S. malayensis (Baling)	154	177	233	225	258	6–8	81	147
	89–205	115–219	177–305	153–279	167–394		51–109	92–194
	±28	±24	±37	±32	±55		±15	±22
S. mekongi	211	212	291	292	288	5–7	73	120
	167–271	167–260	238–349	246–337	149–391		43–115	86–160
	±25	±19	±31	±33	±75		±15	±16
S. japonicum (Philippine)	205	214	279	267	325	6–7	78	134
	122–301	116–298	205–357	242–305	182–465		37–122	86–186
	±38	±36	±39	±19	±78		±20	±25

* Mean.
† Range.
‡ Standard deviation.

TABLE IV. *Comparative measurements of female worms and eggs of* Schistosoma malayensis *(Koyan and Baling strains),* S. mekongi, *and* S. japonicum *(Philippine strain) from mice 50–56 days postinfection.*

Schistosome (strain)	Oral sucker		Ventral sucker		Distance between suckers	Ovary		Embryonated egg*	
	Length	Width	Length	Width		Length	Width	Length	Width
S. malayensis	63†	47	29	36	108	428	102	78	60
(Koyan)	49–90‡	35–66	17–55	25–60	81–164	226–739	44–150	51–104	48–75
	±11§	±8	±9	±8	±20	±99	±23	±8	±5
S. malayensis	54	48	23	28	93	525	129	71	56
(Baling)	39–76	35–58	14–46	16–43	60–141	314–716	95–164	64–85	47–66
	±10	±7	±7	±6	±20	±96	±16	±5	±4
S. mekongi	86	55	37	41	108	431	135	74	60
	58–103	32–65	28–49	32–55	74–155	305–591	108–166	62–97	50–76
	±12	±8	±6	±6	±19	±86	±13	±6	±4
S. japonicum	69	53	37	44	120	525	158	87	62
(Philippine)	44–99	37–69	18–62	28–65	76–185	379–805	81–186	65–103	50–72
	±14	±8	±11	±8	±27	±72	±19	±7	±4

* From liver, 50–52 days postinfection.
† Mean.
‡ Range.
§ Standard deviation.

longed development period for *S. mekongi,* with eggs containing fully developed miracidia first appearing in the liver at 42 days postinfection, 9 days after those of *S. japonicum* (P). For *S. malayensis* (B), fully developed eggs were first seen at 43 days and for *S. malayensis* (K) at 38 days. This rather large difference in developmental rates between strains of *S. malayensis* indicates that this character is not useful as an interspecific taxonomic character.

Important biological differences are seen among the *S. japonicum*-complex schistosomes. It has long been recognized that parasites coevolve with their hosts and one clear expression of this parallel evolution is the development of host specificity. Several studies have demonstrated the development of snail host specificity among the *S. japonicum*-complex schistosomes. Various degrees of compatibility have been demonstrated between geographic strains of *S. japonicum* and subspecies of *Oncomelania hupensis* (Hunter et al., 1952; DeWitt, 1954; Hsu and Hsu, 1960; Moose and Williams, 1963; Chiu, 1965). However, no compatibility was found between *S. mekongi* and 5 subspecies of *O. hupensis* (Lo et al., 1971) or between 5 strains of *S. japonicum* and

TABLE V. *Comparisons of egg size and egg index for isolates of* Schistosoma malayensis, S. mekongi, *and* S. japonicum. *Eggs were recovered from feces of known natural hosts.*

Schistosome (strain or isolate)	Host	Egg size length × width (μm)	Egg index*	Source
S. japonicum (Chinese)	Man	88 × 68	77	Faust and Meleney (1924)†
S. japonicum (Chinese)	Man	76 × 58	76	Faust and Meleney (1924)
S. mekongi (Laos)	Man	61 × 51	84	Barbier (1966)
S. mekongi (Laos)	Man	62 × 51	82	Iijima et al. (1971)
S. mekongi (Laos)	Man	64 × 56	88	Taylor and Moose (1971)
S. mekongi (Laos)	Man	66 × 58	88	Kitikoon (1980)
S. mekongi (Kampuchea)	Man	67 × 56	84	Audebaud et al. (1968)
S. japonicum (Chinese)	Dog	87 × 59‡	68	Hsu and Hsu (1958)
S. japonicum (Formosan)	Dog	90 × 66‡	73	Hsu and Hsu (1958)
S. japonicum (Japanese)	Dog	81 × 64‡	79	Hsu and Hsu (1958)
S. mekongi (Laos)	Dog	65 × 57	88	Kitikoon (1980)
S. malayensis (Koyan)	*R. muelleri*	67 × 54	81	Present report
S. malayensis (Tahan)	*R. muelleri*	67 × 55	82	Present report
S. malayensis (Baling)	*R. muelleri*	65 × 50	77	Present report
S. malayensis (Baling)	*R. tiomanicus*	60 × 49	82	Present report

* Egg index is defined as the ratio of egg width/length × 100.
† These authors presented data for 2 series of egg sizes, large and small. The index was calculated by the present authors.
‡ The average egg sizes from each of 3 dogs presented in the original paper were averaged to obtain the values presented here.

Tricula aperta, snail host for *S. mekongi* (Liang and Kitikoon, 1980). Likewise, Greer et al. (1984b) and Yuan et al. (1984) found no compatibility between strains of *S. japonicum* and *Robertsiella* spp. Conversely, these authors found no compatibility among strains of *S. malayensis* and several subspecies of *O. hupensis.* These results demonstrate that *Oncomelania* spp. are refractory to *S. malayensis* and *S. mekongi* and that snail hosts for the latter 2 species are refractory to *S. japonicum.*

The above reports (Greer et al., 1984b; Yuan et al., 1984) have demonstrated compatibility between some previously untested pairings of snail hosts and schistosomes from continental Southeast Asia. Both reports found *S. malayensis* (K) to be compatible with *T. aperta,* and *S. malayensis* and *S. mekongi* to be compatible with *Tricula bollingi,* a snail not known to be a natural host for any *S. japonicum*-complex schistosome. *Schistosoma mekongi* is incompatible with *R. kaporensis* and one report (Greer et al., 1984b) found *S. mekongi* to be incompatible with *Robertsiella* sp., natural host for *S. malayensis* (B).

Among the Malaysian parasites and hosts, Greer et al. (1984b) reported that *S. malayensis* (K) is compatible with *Robertsiella* sp. but *S. malayensis* (B) is incompatible with *R. kaporensis* and *R. gismanni.*

Additional evidence of biological differences among *S. japonicum*-complex schistosomes is seen in their adaptations to various biotopes. Some of the more readily measurable adaptations are those associated with maintenance of optimal levels of transmission and include, among other factors, differences in cercarial behavior and mammalian host specificity.

A wide variety of mammals has been reported to be natural hosts for *S. japonicum,* particularly in China (Cheng, 1971). The snail hosts, *O. hupensis,* are amphibious and have been recovered from a variety of pristine habitats including mountain streams (Hsu and Hsu, 1967), river marshes (Ritchie et al., 1953), and virgin forest streams in alluvial plains (Pesigan et al., 1958). In addition, all subspecies of *O. hupensis* have adapted well to a variety of man-altered water systems, notably those associated with rice agriculture. Although there are conflicting reports as to the time of cercarial emergence, strong evidence has been presented showing that they emerge soon after dried snails are submerged, regardless of the time of day (Pesigan et al., 1958; Nojima et al., 1980), or during daylight hours for snails that remain submerged (Mao et al., 1949; Pesigan et al., 1958; Nojima et al., 1980).

TABLE VI. *Comparisons of egg size and egg index of isolates of* Schistosoma malayensis, S. mekongi, *and* S. japonicum. *Eggs were obtained from experimentally infected laboratory mice.*

Schistosome (strain or isolate)	Egg size length × width (μm)	Egg index*	Source
S. japonicum (Chinese)	87 × 58†	67	Hsu and Hsu (1958)
S. japonicum (Formosan)	84 × 60†	71	Hsu and Hsu (1958)
S. japonicum (Japanese)	81 × 61†	75	Hsu and Hsu (1958)
S. japonicum (Philippine)	84 × 59†	70	Hsu and Hsu (1958)
S. japonicum (Chinese)	72 × 48‡	67	Voge et al. (1978)
S. japonicum (Formosan)	67 × 49‡	73	Voge et al. (1978)
S. japonicum (Japanese)	64 × 48‡	75	Voge et al. (1978)
S. japonicum (Philippine)	67 × 50‡	75	Voge et al. (1978)
S. japonicum (Philippine)	87 × 62§	71	Present report
S. mekongi (Laos)	55 × 37‡	67	Voge et al. (1978)
S. mekongi (Laos)	74 × 60§	81	Present report
S. malayensis (Koyan)	78 × 60§	77	Present report
S. malayensis (Baling)	71 × 56§	79	Present report

* Egg index is defined as the ratio of egg width/length × 100.
† Eggs recovered from feces of mice.
‡ Eggs recovered from mouse liver at 35 days postinfection.
§ Eggs recovered from mouse liver at 50–52 days postinfection.

Unlike the wide range of mammalian hosts and diversity of biotopes known for *S. japonicum* transmission, *S. mekongi* and *S. malayensis* have been reported from relatively few species of mammals and occur in a relatively narrow range of biotopes. Man and dog are the only known natural mammalian hosts for *S. mekongi* (Sornmani et al., 1980), even though a number of pigs, cattle, wild rodents (Kitikoon et al., 1975), and buffalo (Schneider et al., 1975) have been examined from endemic areas. *Tricula aperta* is a completely aquatic snail with a rather limited distribution, being found only in the Mekong and Mun rivers near the Thai–Laos border and in the Mekong River as far south as Kratie, Kampuchea (Davis et al., 1976; Kitikoon and Schneider, 1976). Those rivers are relatively large and swift in regions where the snails occur. *Schistosoma mekongi* cercariae emerge during the early morning (Lohachit et al., 1980), indicating a predominately daytime transmission.

Man and wild rats are the only known natural hosts for *S. malayensis* (Ambu et al., 1984). But the low prevalence in man, combined with the failure to recover eggs from the stool of a biopsy-positive patient (Murugasu et al., 1978) or from serologically positive patients (Greer and Anuar, 1984), suggest that man is not an important host for this parasite. A small number of experimental infections indicate that dogs also are not suitable

hosts for *S. malayensis* (Ambu et al., 1984). Species of *Robertsiella* are strictly aquatic, have a narrow habitat range, and have been found only in small streams in the foothills and mountainous regions of Peninsular Malaysia (Greer et al., 1984a). Cercariae emerge in late afternoon and night (Greer and Ow-Yang, 1985) indicating a transmission that is predominately crepuscular and nocturnal.

Differences in isoenzyme migration patterns offer a more direct measure of genetic differences. Several recent studies have demonstrated magnitudes of difference among isolates of *S. malayensis, S. mekongi,* and *S. japonicum* that are thought to be representative of differences among separate species. Fletcher et al. (1980), from a study of electrophoretic migrations of 11–12 isoenzymes, presented strong evidence for the separation of *S. mekongi* and *S. japonicum.* Those authors reported distinct differences between *S. mekongi* and 4 strains of *S. japonicum* that ranged from 82 to 91%, while differences among the *S. japonicum* strains ranged from 17 to 36%.

Yong et al. (1985) demonstrated significant genetic differences among *S. malayensis, S. mekongi,* and *S. japonicum. Schistosoma malayensis* (K) was found to be distinctly different from *S. japonicum* in 10 of 11 isoenzymes and it differed from *S. mekongi* in 5 of 11 isoenzymes. The latter difference is equivalent to a Nei's (1972) mean genetic distance of 0.607, which is similar to distances reported among sibling species and for some distinct invertebrate species (Ferguson, 1980). Results from 9 isoenzymes indicated no differences between *S. malayensis* Baling and Koyan strains (Yong et al., 1985).

Viyanant and Upatham (1985) reported differences between *S. malayensis* (K) and *S. mekongi* in 4 of 10 isoenzymes separated by isoelectric focusing. None of these differences were reported in Yong et al. (1985), thus when duplications are taken into account, the combined results from these studies show that the 2 species differ in 9 of 16 isoenzymes tested.

These genetic comparisons and studies on snail host specificity clearly demonstrate a closer affinity between *S. mekongi* and the Malaysian schistosome than between either and *S. japonicum.* The discussion above presents further support for the separation of *S. mekongi* from *S. japonicum.* Furthermore, we believe the results from these various studies demonstrate species level differences between *S. malayensis* and its closest congener *S*

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RAILLIETIELLA TEAGUESELFI N. SP. (PENTASTOMIDA: CEPHALOBAENIDA) FROM THE MEDITERRANEAN GECKO, *HEMIDACTYLUS TURCICUS* (SAURIA: GEKKONIDAE), IN TEXAS

John Riley, Chris T. McAllister*, and Paul S. Freed†
Department of Biological Sciences, The University, Dundee DD1 4HN, Scotland

ABSTRACT: *Raillietiella teagueselfi* n. sp. is described from the lungs of the Mediterranean gecko, *Hemidactylus turcicus*, in Houston, Texas, U.S.A. This new pentastomid is most closely related to *R. affinis* Bovien, 1927, of Asiatic Tokay geckos (*Gekko gecko*) in Indonesia, but mature males differ in possessing rounded posterior hooks with swollen, blunt-tipped barbs, instead of relatively sharp-tipped hooks. In addition, the annulus number of females can readily differentiate the new species from 6 of the 10 blunt-tipped species (*R. gehyrae* Bovien, 1927; *R. hemidactyli* Hett, 1934; *R. maculatus* Rao and Hiregauder, 1959; *R. monarchus* Ali et al., 1984; *R. mabuiae* (Heymons, 1922); and *R. scincoides* Ali et al., 1984). Two of the remaining 3 species of blunt-tipped raillietiellids (*R. frenatus* Ali et al., 1981, and *R. maculibris* Ali et al., 1984) are differentiated from female *R. teagueselfi* by having larger hook dimensions. The new species can be distinguished from *R. freitasi* (Motta and Gomes, 1968) by having a hooked spicule. Observations on the prevalence and intensity of infections are presented.

Pentastomids belonging to the genus *Raillietiella* Sambon, 1910, infect the lungs of a variety of reptiles, including lizards, amphisbaenians, and snakes, and more recently, certain amphibians (toads) have been confirmed as definitive hosts (Ali et al., 1982c). Four species of *Raillietiella* have been described from gekkonid lizards. It is particularly important that raillietiellids that infect geckos be well characterized, because many species have been widely introduced through commerce via shipping routes into coastal and island port cities (Self and Diaz-Garcia, 1961; Ali et al., 1984b). Further, under experimental circumstances, some raillietiellids are capable of maturing in gecko hosts that are not naturally infected (Lim and Yong, 1977; Ali and Riley, 1983; Bosch, 1986). Thus, it appears that ecological rather than physiological factors govern host specificity.

Less than a century ago, the Mediterranean gecko, *Hemidactylus turcicus* (Linnaeus, 1758), was inadvertantly introduced into the United States and has become a very successful colonizer (Selcer, 1986). Ranging naturally from the Mediterranean basin of Spain and Morocco east to Egypt and the Persian Gulf, and south to Somalia, the species is now well established throughout the Gulf of Mexico, from peninsular Florida west to Texas, and south along coastal Mexico to Panama (Loveridge, 1941; McCoy, 1970). Dixon (1987) reported that *H. turcicus* is continuing to spread rapidly over Texas and has been found in 41 counties since its discovery in 1955 in Cameron County.

During a parasitological survey of *H. turcicus*, we found numerous pentastomids in the lungs of geckos from Houston, Texas. Further examination revealed that the specimens represented a previously undescribed species of cephalobaenid pentastomid. Herein, we provide a description of the new form, along with observations on its prevalence and intensity.

MATERIALS AND METHODS

Between December 1986 and June 1987, 86 (45 males, $\bar{x} \pm$ SD snout–vent length = 46.9 ± 7.5, range 28–58 mm; 41 females, 47.0 ± 7.5, range 32–56) hatchling, juvenile, and adult *H. turcicus* were collected by hand from within the reptile and amphibian facility at the Houston Zoological Gardens (n = 57) and on the walls of St. Anne's Catholic Church (n = 22) in Houston, Harris County, Texas, and in a private residence (n = 7) in Houma, Terrabonne Parish, Louisiana. At the former locality, the geckos roam free and are not part of the captive fauna of the zoo. Within 48 hr, geckos were killed by sodium pentobarbital overdose and examined for parasites. Pentastomids (primary larvae, nymphs, and adults) taken from the lungs were fixed immediately, while alive, in 70% ethanol. The body length of ethanol-fixed females was measured and

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* To whom correspondence and reprint requests be addressed: Department of Biological Sciences, North Texas State University, Denton, Texas 76203 and Renal-Metabolic Lab (151-G), Veterans Administration Medical Center, 4500 S. Lancaster Road, Dallas, Texas 75216.

† Department of Herpetology, Houston Zoological Gardens, 1513 Outer Belt Drive, Houston, Texas 77030.

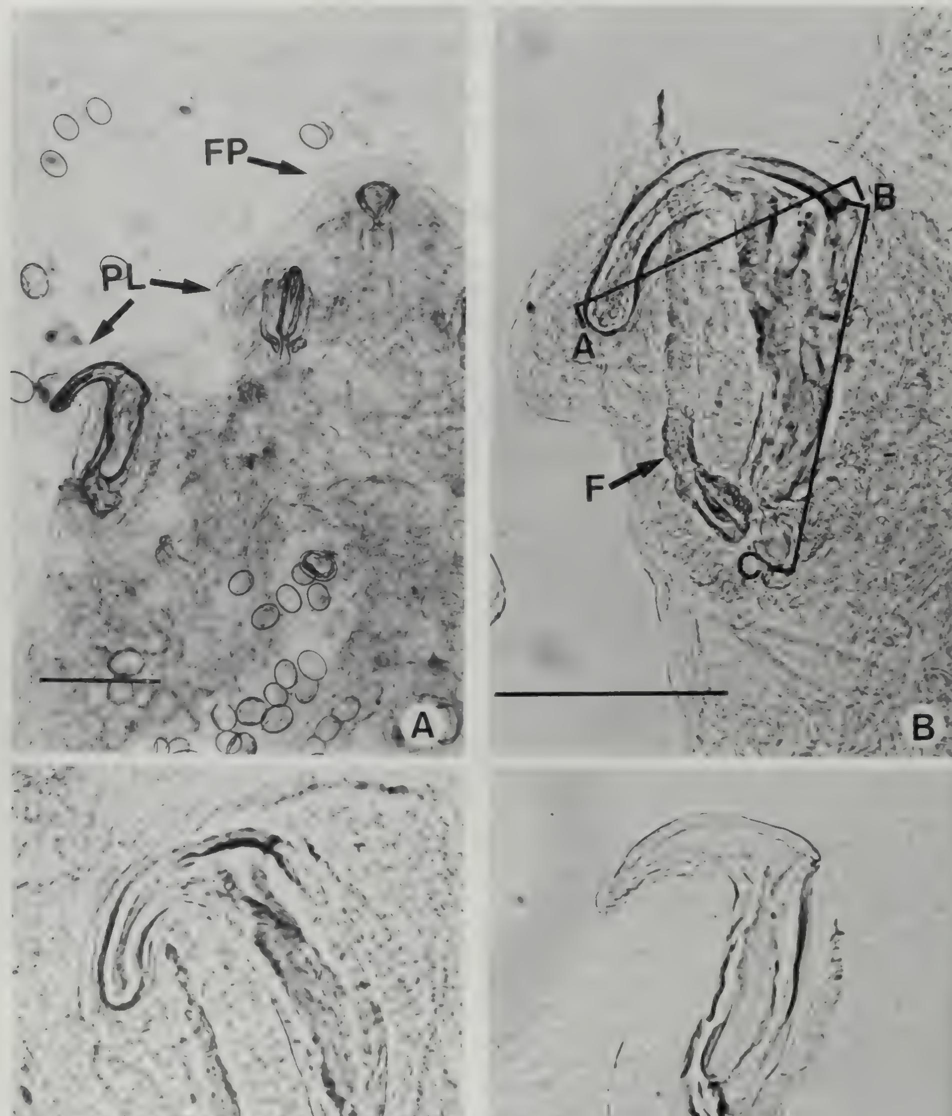

FIGURE 1. *Raillietiella teagueselfi* n. sp. and *R. affinis*. **A.** Cephalothorax of a gravid female *R. teagueselfi* showing the disposition of the mouth and hooks. The anterior triangular mouth is flanked by a pair of domed frontal papillae (FP) (Ali and Riley, 1985), and the hooks are flanked by parapodial lobes (PL). The fulcrum supporting the small anterior hook is clearly visible as is the relatively massive, bluntly rounded posterior hook. Bar = 400 μm. **B.** Detail of a paratype female posterior hook supported by the fulcrum (F). The 2 parameters measured (AB = the distance from the hook tip to the notch on the hook shank and BC = the distance from

the abdominal annuli counted. The cephalothorax was then removed, mounted in Hoyer's medium, and flattened under gentle coverslip pressure so that hooks could be correctly measured. A series of the smaller males was mounted whole in Hoyer's medium for all measurements. Specimens retained for SEM were either fixed for prolonged periods in 70% ethanol or 4% glutaraldehyde and washed in buffer before being critical-point dried. Seventeen mature females and 12 males were used for each measurement; measurements are presented in μm, unless otherwise stated, and are reported as means followed by the ranges in parentheses. Representative specimens are deposited in the USNM Helminthological Collection, USDA, Beltsville, Maryland 20705, U.S.A.

DESCRIPTION

Raillietiella teagueselfi n. sp.

(Figs. 1–3)

Raillietiella teagueselfi n. sp. with the characteristics of the genus, Sambon, 1910.

Female: Holotype 12 mm long, with 23 annuli; paratype series (n = 16), 11.13 (9–14) mm long, with 23.6 (23–25) annuli. Anterior genital pore associated with second abdominal annulus; first abdominal annulus delimited posteriorly by narrow depression below posterior hooks. Most other (<24) abdominal annuli easily visualized as discrete raised tiers of cuticle. Anus terminal, caudal segment bifurcated. Anterior hooks sharp-pointed, posterior hooks much larger with very obvious bluntly rounded tips, which in some specimens are swollen (Fig. 1A, B); dimensions AB 258 (220–295), BC 355 (335–390). A proportion (undetermined) of all eggs in saccate uterus contain fully developed, hooked, primary larvae, all most likely mature and patent (Ali et al., 1984b).

Male: Paratype series (n = 12), 4.58 (3–6) mm long, with 19.4 (18–21) annuli; posterior hook bluntly rounded as in female; dimensions AB 140 (120–155), BC 205 (190–210) (Fig. 1C). Body widest in anterior half of abdomen, tapered posteriorly, giving pronounced claviform appearance (Fig. 3). Copulatory spicules with curved shaft; thickened base flared and hooked but largely devoid of ornamentation (Fig. 2A). Overall spicule length 330 (305–360), maximum base width 110 (105–125).

Holotype: USNM Helm. Coll. No. 80026.

Paratypes: USNM Helm. Coll. No. 80027 (n = 28). Immature females USNM Helm. Coll. No. 80028 (n = 4). Additional specimens USNM Helm. Coll. No. 80028 (n = 25) (in 70% ethanol).

Type host: *Hemidactylus turcicus turcicus* (Linnaeus, 1758), Mediterranean gecko.

Type locality: U.S.A.: Texas: Harris Co.: Houston, Zoological Gardens.

Prevalence: 17/86 (19.8%) of the *H. turcicus* were infected. However, only adult geckos (51.3 ± 3.6, 45–56 mm) from Houston Zoo site (17/57 = 29.8%) harbored *R. teagueselfi*. There were no significant sexual differences in prevalence of pentastomids (9/32 = 28.1% males versus 8/25 = 32.0% females; $\chi^2 = 0.075$, $P > 0.05$, df = 1).

Intensity: Range 1–193 ($\bar{x} = 18.5$).

Site of infection: Lungs.

Etymology: Named in honor of Dr. J. Teague Self, Emeritus Professor of Zoology, University of Oklahoma, in recognition of his numerous contributions to understanding the biology of the Pentastomida.

Remarks: The morphology of *R. teagueselfi* n. sp. is closest to *R. affinis* Bovien, 1927, a species infecting the Tokay gecko, *Gekko gecko* in Indonesia. For comparative purposes, measurements of 3 slide-mounted males (USNM Helm. Coll. No. 80029) of this species from Bogor, Java, Indonesia, are included in this report.

DISCUSSION

Fixed raillietiellids possess comparatively few good characters upon which to establish specific diagnosis. Nonetheless, appropriate combinations of body length, annulus number, and posterior hook dimensions, together with the shape and size of the male copulatory spicule, have provided adequate criteria for the most recent studies of the systematics of the group (Ali et al., 1981, 1982a, 1982b, 1982c, 1984a, 1984b, 1985). However, there remains a dearth of supporting experimental life-cycle studies (Ali and Riley, 1983; Jeffery et al., 1985; Bosch, 1986).

In the latest review of the genus, Ali et al. (1985) assorted the 24 currently recognized species (i.e., at least 7 others await confirmation of suspected species status pending additional material) into 5 taxa based primarily on host differences. A total of 14 species infect small insectivorous lizards and these further subdivide naturally into 2 groups on the basis of marked differences in the morphology of the posterior hook, which is either sharply pointed (Group I) or very bluntly rounded (Group II). This concept was originally presented by Self (1969).

The characteristics of the 10 species in Group II are listed in Ali et al. (1985); 4 parasitize gekkonid lizards, 5 occur in scincoid lizards, and the remaining species is found in *Calotes versicolor* (family Agamidae).

←

the notch to the hook tip are indicated). Bar = 250 μm. **C.** Detail of the posterior hook of a male *R. teagueselfi* n. sp. showing the swollen, blunt tip to the barb. Bar = 100 μm. **D.** The posterior hook of a male *R. affinis* from *Gekko gecko*. The relatively sharp tip to the hook is clearly visible seen underneath a fleshy sheath that covers the hook. Bar = 100 μm.

FIGURE 2. Copulatory spicules. **A.** Copulatory spicule of a cleared *R. teagueselfi* n. sp. showing the curved shafts and prominent, hooked, flared bases. The "hooks" of both spicules face left in this specimen and the distinguishing projection on the flared base, opposite the hook, is shown (arrows). Bar = 200 μm. **B.** Spicule of *R. affinis.* Although the base is hooked (this is less pronounced than in *R. teagueselfi*) the projection is demonstrably absent. Bar = 100 μm.

It is relatively straightforward to differentiate *R. teagueselfi* from other blunt-hooked species, even though we are only able to measure relatively few characters. Consider just 2, annulus number and hook size.

The annulus number of females alone can differentiate *R. teagueselfi* from 6 of the 10 blunt-hooked species. Thus, *R. gehyrae* Bovien, 1927 (21–22), *R. hemidactyli* Hett, 1934 (26–30), *R. maculatus* Rao and Hiregauder, 1959 (28–33), *R. monarchus* Ali et al., 1984b (28–30), *R. mabuiae* (Heymons, 1922) (28–37), and *R. scincoides* Ali et al., 1984b (33) are clearly unrelated. There are, however, degrees of overlap in annulus counts when we compare data from Ali et al. (1985) between the new species and *R. frenatus* Ali et al., 1981 (23–27), *R. maculiabris* Ali et al., 1984b (24–28), *R. freitasi* (Motta and Gomes, 1968) (23–25), and *R. affinis* (23–26). However, female hook dimensions of *R. teagueselfi* are smaller than those of the first 2 species in the latter group (e.g., *R. frenatus* AB 345–400, BC 485–550; *R. maculiabris* 305–325, 415–455; Ali et al., 1985). This leaves, after comparisons of just 2 characters, the possibility that *R. affinis* from *G. gecko* in Java, Indonesia and *R. freitasi* from *Mabuya punctata* in Brazil may be related to *R. teagueselfi*; however, this represents an unlikely prospect due to marked differences in host zoogeography

Indeed, as we will show, when respective males are compared, this possibility can readily be discounted. Ali et al. (1982a) in their reappraisal of the taxonomic status of *R. affinis* found that although the posterior hook of gravid and maturing females was blunt-tipped, hooks of mature males were sharp-tipped. For example, Figure 1D illustrates such a hook from an adult, 4.5-mm-long male, taken from Bogor, Java, compared to a typical *R. teagueselfi* hook (Fig. 1C) with its obviously rounded, slightly swollen tip. The spicules of *R. affinis*, based on 3 specimens, measure 330–360 in length and 110–120 across the base but, as shown in Ali et al. (1985, fig. 2) and Figure 2B, the base is hooked toward the inner curvature of the shaft but is rounded elsewhere. By contrast, a consistent feature of the spicule of *R. teagueselfi* is a small projection on the opposite side of the hook on the base, a situation known in only one other blunt-hooked species, *R. monarchus* from Malaysian *G. monarchus* (Ali et al., 1984b, 1985). The spicule of *R. freitasi* is not hooked but possesses a bluntly rounded base (Ali et al., 1984b). This character-

FIGURE 3. SEM of *R. teagueselfi* n. sp. **A.** Stereoscan of an entire male *R. teagueselfi* from the ventral aspect showing the relatively tiny cephalothorax united with a massive annulated abdomen. Note the dorsal papillae (arrow) and the genital pore (arrowhead), which lies between annuli 2 and 3. Bar = 500 μm. **B.** Detail of the cephalothorax and anterior abdomen of a male showing the relative dispositions of the mouth and hooks. Bar = 100 μm.

istic immediately differentiates the species from *R. teagueselfi.*

Recently, Pence and Selcer (1988) reported that 44% of *H. turcicus* from Edinburg, Hidalgo County, Texas, was infected with the pentastomid *R. frenatus.* Further, the authors noted that *R. frenatus* occurred in gecko populations in the lower Rio Grande Valley of Texas, and in Tampa, Miami, and Key West, Florida, but not in populations of *H. turcicus* surveyed from Corpus Christi and Galveston, Texas, New Orleans, Louisiana, and Gainesville, Florida. The data suggest that pentastomids may occur only in southern populations of *H. turcicus* (i.e., below the freeze line) but apparently not in the more northern populations (i.e., above the freeze line). This freeze-line hypothesis is seductive in that climate may play a role in the distribution of

some (but not all) species of Pentastomida in the New World. Indeed, in the present study, the moderately high prevalence and intensity of infections in a localized population of this free-ranging host implies that *R. teagueselfi* has, relatively recently, adapted to a novel insect intermediate host (Ali and Riley, 1983; Jeffery et al., 1985; Bosch, 1986). In addition, because *R. teagueselfi* parasitized geckos only in the Houston Zoo locality and not in a nearby study site (St. Anne's Church) suggests that other factors, and not climate, may be operating to maintain the transmission of this pentastomid parasite. From this point of view alone, the new species is more than unusually interesting, and further study into its overall distribution is warranted.

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INERMICAPSIFER BEVERIDGEI N. SP. (CESTOIDEA: ANOPLOCEPHALIDAE) FROM *PROCAVIA CAPENSIS* (HYRACOIDEA) IN ISRAEL, WITH NOTES ON TWO SPECIES OF *HYMENOLEPIS*

Gerald D. Schmidt and Guta Wertheim*
Department of Biological Sciences, University of Northern Colorado, Greeley, Colorado 80639

ABSTRACT: *Inermicapsifer beveridgei* n. sp. from *Procavia capensis* differs from all other species in the genus in having 150–174 testes per segment. The closest species is *I. hyracis* (Rudolphi, 1810) Janicki, 1919, with 75–120 testes. *Hymenolepis vogeae* Singh, 1956 and *H. horrida* (Linstow, 1901) Lühe, 1910, are briefly discussed.

In a previous publication (Wertheim et al., 1986) survey data on cestodes of small mammals from Israel and the Sinai peninsula were presented. In that paper *Inermicapsifer* sp. was reported from *Procavia capensis,* with the comment that the species might be new. The present report describes that species. The specimens were fixed, stained with Semichon's carmine, and mounted by conventional technique. Measurements are in μm unless otherwise indicated.

Inermicapsifer beveridgei n. sp.
(Figs. 1–3)

Seven gravid specimens, collected from 3 rock hyrax, *Procavia capensis,* in Gilboa Mountains, Israel, represent a previously undescribed species.

Description

Strobila 94–133 mm long, 3.5–4.0 mm greatest width at posterior end (n = 7). Scolex (Fig. 1) 950–1,000 long, 825–850 greatest width (n = 7). Suckers rounded, 260–280 wide (n = 10). Neck variable according to state of relaxation, 1.0–1.85 mm long (n = 7).

Proglottids craspedote. Genital pores unilateral, postequatorial. Genital ducts (Fig. 2) pass between osmoregulatory canals. Genital atrium simple, 15–25 deep, 75–85 long (n = 10). Ventral osmoregulatory canals with simple anastomosis near posterior end of each proglottid. Ventral canals about 20 wide, dorsal reticulated, about 5 wide in mature segments. Reproductive systems (Fig. 2) mature at about the same time.

Male genitalia: 150–174 testes in single field posterior and lateral to ovary (n = 20); each 40–55 wide in mature segments (n = 50). Vas deferens coiled in posterior, poral field. Ejaculatory duct coiled inside cirrus pouch, enlarged distally to form internal seminal vesicle. Cirrus pouch elongated oval; 280–640 long, 110–175 greatest width (n = 20), increasing size as proglottid ages. Cirrus thick, unarmed.

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* Laboratory of Helminthology, Hebrew University–Hadassah Medical School, Jerusalem, Israel.

Female genitalia: Ovary poral, lobulated, 240–360 long, 270–280 wide (n = 20). Vitellarium compact, posteromedial to ovary, 115–125 long, 140–160 wide (n = 20). Vagina posterior to cirrus pouch, muscular, sometimes twisted. Seminal receptacle with terminal sac 100–150 long, 70–90 wide (n = 10). About 200 egg capsules per segment (Fig. 3) (n = 10), each 150–200 wide, containing 4–10 eggs each (n = 20). Eggs 35–50 wide (n = 25). Onchospheres about 20 wide; hooks not observed.

Type host: Rock hyrax, *Procavia capensis* Pallas, 1766.

Type locality: Gilboa Mountains, Israel.

Habitat: Small intestine.

Type specimens: Hebrew University, Jerusalem, Parasitological Collection: holotype no. 1675a, paratypes no. C703, C751, 1675.

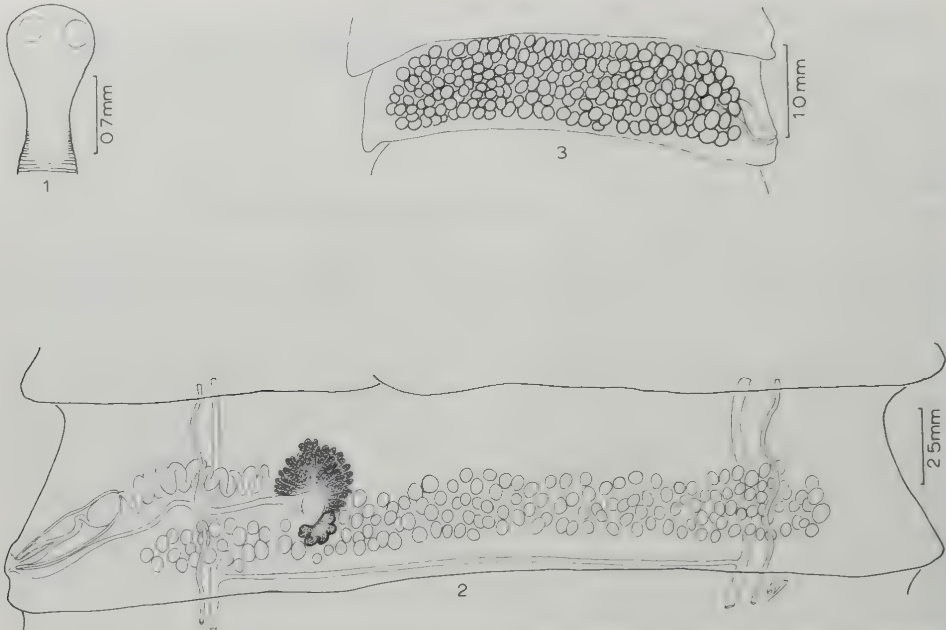
Etymology: Named in honor of Dr. Ian Beveridge, Institute of Medical and Veterinary Science, Adelaide, Australia, who contributed much to our knowledge of the Anoplocephalidae.

Taxonomic summary

Inermicapsifer beveridgei n. sp. differs from all other species in the genus in having many more testes per segment (150–174). Of those nominal species with testes in 1 field, *I. hyracis* (Rudolphi, 1810) Janicki, 1919, has the greatest number, with 75–120 according to various authors (Janicki, 1910; Baer, 1927; Mahon, 1954). Other species of *Inermicapsifer* from *Procavia capensis* with 1 field of testes, with their testes numbers, are: *I. interpositus* Janicki, 1910 (48–80), and *I. sinaitica* (Bischoff, 1912) Spasskii, 1951 (60–80). Thus, the number of testes in the present specimens clearly indicates a separate and new species.

Hymenolepis vogeae Singh, 1956

This species was found in *Gerbillus tristrami* Thomas, 1892 (Cricetidae), at Mishmar Ha'Emek and in *Skeetemys calurus* Thomas, 1892 (Cricetidae) at Gebel Sorbal, the Sinai. It was reported as *Hymenolepis* sp. by Wertheim et al. (1986). It appears to show little host specificity, as it was originally described from *Mus buduga* (Muridae) in India by Singh (1956); subsequently it was redescribed from *Meriones libycus* (Cricetidae) from Egypt by Mikhail and Fahmy



FIGURES 1–3. *Inermicapsifer beveridgei* n. sp. from rock hyrax in Israel. **1.** Scolex. **2.** Mature segment. **3.** Gravid segment, showing egg capsules.

(1976). Our specimens also match very closely with *H. uranomidis* Hunkeler, 1972, from several murid species in West Africa, except that the glandular cells surrounding the external seminal vesicle described by Hunkeler (1974) could not be seen.

Voucher specimens are deposited in HUJP no. S382, 465.

Hymenolepis horrida (Linstow, 1901) Lühe, 1910

This species has wide distribution in Europe and North America, in several species of rodents. We found it in 4 *Meriones tristrami* at Mishmar Ha'Emek, Sinai, which constitutes both new host and distribution records. It was reported as *Hymenolepis* sp. by Wertheim et al. (1986).

Voucher specimens are deposited in HUJP no. 465.

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RESEARCH NOTES

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Destruction and Regrowth of Testes in *Philophthalmus gralli*, an Eyefluke of Birds

Alain W. Vilatte and Paul M. Nollen, Department of Biological Sciences, Western Illinois University, Macomb, Illinois 61455

ABSTRACT: Adults of *Philophthalmus gralli* were subjected to deionized water at room temperature and 3 C. Destruction of the stages of spermatogenesis in the testes was observed after 3 hr of exposure at room temperature. Complete regrowth of normal stages of spermatogenesis was found in worms transplanted back to the host for 10 days.

The ability of digenetic trematodes to regenerate complete body parts and organs is almost nonexistent when compared to the turbellarians. Previous studies have indicated that, outside of wound healing, the digenetic trematodes have little regenerative ability (Fried and Penner, 1964; Fried et al., 1971; Austin and Fried, 1972; Bentley, 1982; Popiel et al., 1985).

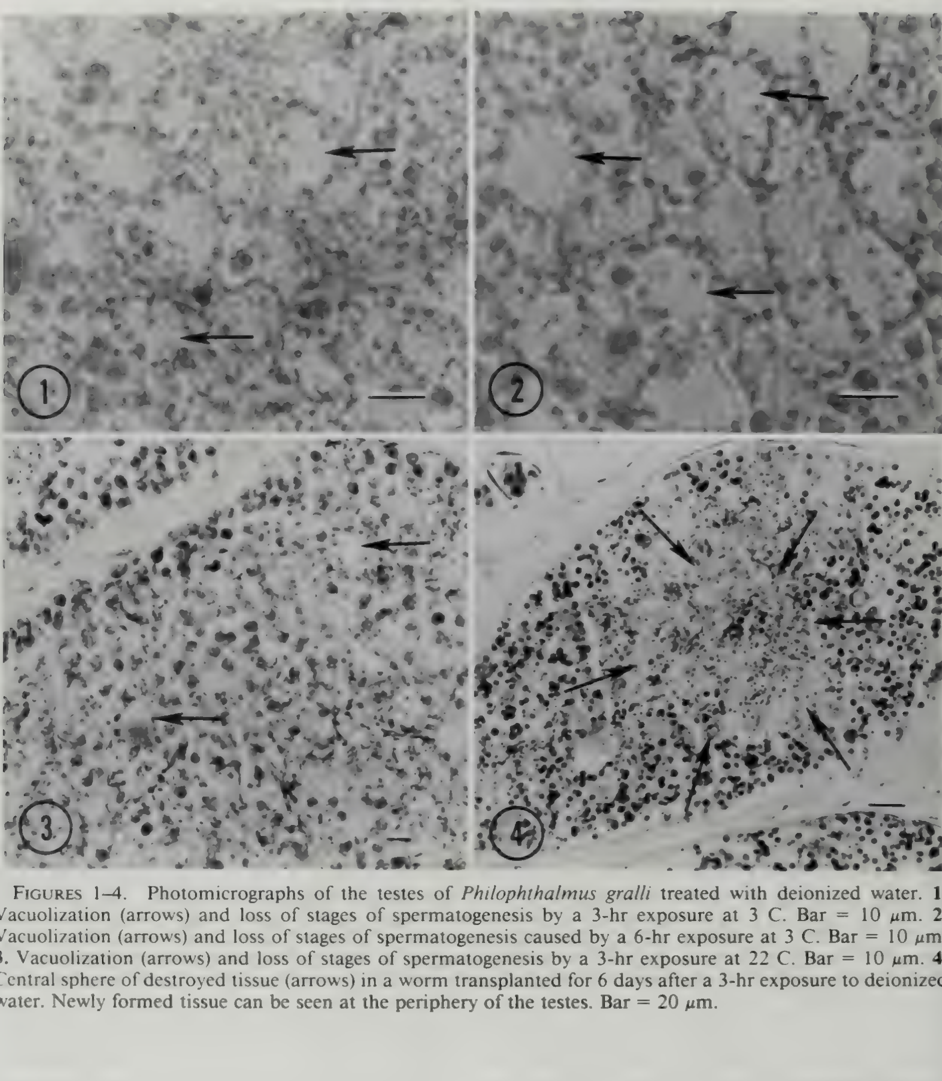
Reproductive organs of digenetic trematodes are the most sensitive tissues to suboptimal conditions and are the first to be reabsorbed or destroyed. Nollen and Alberico (1972) suggested that the poor nutritional conditions of frogs stored in holding facilities before sale by commercial suppliers caused their bladder flukes to lose their testes. The suboptimal nutritional conditions of *in vitro* culture media for schistosomes caused progressive loss of ovaries, testes, and vitelline glands (Michaels, 1969; Floyd and Nollen, 1977; Kolzow and Nollen, 1978; Irie et al., 1987). Exposure of adults to deionized water will also disrupt the testes as was found in a study with the eyefluke, *Philophthalmus gralli* (MacNab and Nollen, 1987).

Can the reproductive organs initiate production of the stages of spermatogenesis when the flukes are returned to normal nutritional and osmotic conditions, or is the damage permanent? When pairs of *Schistosoma mansoni* grown *in vitro* for long periods of time and with impaired reproductive systems were transplanted to mice, they regained the ability to produce normal eggs (Basch and Humbert, 1981). This indicated that the male and female could regenerate reproductive tissues or recover from a damaged condition. The testes of *P. gralli* are severely disrupted with no recognizable stages of spermatogenesis when treated with deionized water for several hours. We investigated this problem by subjecting adults of *P. gralli* to various osmotic conditions and different temperatures to determine the most important factor in the tissue destruction. Worms with impaired testes were then transplanted to chickens to see if regrowth of the testicular tissue would take place and, if so, how fast.

Gravid adult *P. gralli* (40–60 days old) were harvested from chickens, placed in small petri dishes containing deionized water at 3 C, and removed in groups of 5 at hourly intervals for 6 hr. A second trial repeated this procedure at 22 C. A third trial involved immersing flukes in 0.85% saline (NaCl) at 22 C. Worms for transplantation were kept in deionized water for 3 hr at 22 C and then placed in Hedon Fleig's saline (Dawes, 1954) and allowed to warm gradually to 39 C for 2 hr. These worms were then transplanted on polished glass rods in groups of 4 per eye directly to the orbits of uninfected chickens of the same age as the original hosts. The worms were then harvested at daily intervals up to 10 days. All resulting flukes were prepared for paraffin sectioning, stained with hematoxylin and eosin, and observed for histological abnormalities and evidence of the production of normal stages of spermatogenesis.

The testes of worms exposed to deionized water at 3 C showed signs of vacuolization after 3 hr (Fig. 1). A progressive loss of the stages of spermatogenesis was noted in the 4–6-hr specimens (Fig. 2). The vitellaria and ovaries of these worms were unaffected by treatment with cold deionized water up to 6 hr.

In those worms subjected to deionized water at 22 C, the disruption of the testes was much faster, and by 3 hr no stages of spermatogenesis remained and large vacuoles were observed (Fig. 3). Worms immersed in 0.85% saline at 22 C were normal in morphology of tissues for the 6-hr duration of the trial. This series of experi-



FIGURES 1–4. Photomicrographs of the testes of *Philophthalmus gralli* treated with deionized water. **1.** Vacuolization (arrows) and loss of stages of spermatogenesis by a 3-hr exposure at 3 C. Bar = 10 μm. **2.** Vacuolization (arrows) and loss of stages of spermatogenesis caused by a 6-hr exposure at 3 C. Bar = 10 μm. **3.** Vacuolization (arrows) and loss of stages of spermatogenesis by a 3-hr exposure at 22 C. Bar = 10 μm. **4.** Central sphere of destroyed tissue (arrows) in a worm transplanted for 6 days after a 3-hr exposure to deionized water. Newly formed tissue can be seen at the periphery of the testes. Bar = 20 μm.

ments points to the hypotonic nature of the medium as the cause of the disruption of tissues.

Worms exposed to deionized water at 22 C for 3 hr and transplanted to chickens showed signs of testicular regrowth after 2 days. This renewal of tissue originated at the periphery of the testes. Evidently the stem cells that produce the primary spermatogonia at the periphery of the testes were not destroyed by the hypotonic conditions and were able to continue normal cell division with the restoration of optimal conditions. This pattern of regrowth left distinct spheres of damaged tissue at the center of the testes (Fig. 4). By 10 days after transplantation the central spheres were gone and the testes had completely returned to the undamaged state. This is further evidence that the reproductive organs of digenetic trematodes are not completely destroyed by adverse conditions and can initiate regrowth of normal structure when returned to normal nutritional and osmotic conditions.

The morphology and function of stem cells needs further study at the ultrastructural level in digenetic trematodes. The paraffin sections from this study do not give details of structure at the periphery of the testes. Are the stem cells de-

stroyed and testicular tissue newly produced from nongerminal cells, or are the stem cells left undamaged by the hypotonic treatment and do they start to divide to produce spermatogonia when conditions return to normal?

Although the stages of spermatogenesis have been studied ultrastructurally in several species, much less is known about the peripheral area of the testes (Grant et al., 1976; Robinson and Halton, 1982; Erwin and Halton, 1983). Two types of cells have been found in the peripheral region of the testes, mainly supportive cells called either sustentacular or nutritive cells and primary spermatogonia. However, no differentiation has been made between the first cell of spermatogenesis, the primary spermatogonium, and the stem cells that divide to produce the primary spermatogonia. An ultrastructural study would be in order to see what cells are left at the periphery of the testes after hypotonic disruption, and if stem cells and primary spermatogonia can be differentiated.

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Time-Course of *Giardia muris* Infection in Male and Female Immunocompetent Mice

Martin F. Heyworth, Cell Biology Section (151E), Veterans Administration Medical Center, San Francisco, California 94121, and Department of Medicine, University of California, San Francisco

ABSTRACT: The time-course of acute *Giardia muris* infection was compared in male and female immunocompetent BALB/c mice that had not previously been exposed to the parasite. No sex-related difference was observed in the time-course of the infection in these mice. Sexually mature mice of both sexes excreted substantial numbers of *G. muris* cysts ($>10^4$/2 hr) over a longer period than did sexually immature mice.

Sex-related differences in immune responsiveness have been observed in various experimental animals. Such differences may be due to genetic factors or may reflect the influence of sex hormones on immunological responses. In many cases, immune responsiveness is greater in females than in males. For example, the severity of *Streptococcus pyogenes* infection in male mice exceeds that observed in female mice, and the mortality of rabbits infected with a toxic shock strain of *Staphylococcus aureus* is lower for females than for males (Willoughby and Watson, 1964; Best et al., 1984). On the other hand, it has been found that estrogen administration increases the severity of *Listeria monocytogenes* infection in mice and of *Toxoplasma gondii* infection in guinea pigs (Kittas and Henry, 1979; Pung et al., 1984).

The aim of the present work was to monitor the time-course of *Giardia muris* infection in male and female immunocompetent BALB/c mice that had not previously been exposed to the parasite, to determine whether there is a sex-related difference in the ability of such mice to clear this intestinal protozoan infection. Cesarian-originated, barrier-reared male and female BALB/c mice were obtained from Charles River Laboratories, Kingston, New York. These mice were housed in the Animal Care Facility at the San Francisco V.A. Medical Center and were fed on Purina laboratory chow and water *ad libitum*. Before mice were infected with *G. muris*, fecal specimens from these animals were examined for *G. muris* cysts, using the method summarized in the next paragraph (Roberts-Thomson et al., 1976). No *Giardia* cysts were detected in fecal samples obtained before experimental infection of mice with *G. muris*.

Mice were infected with *G. muris* by peroral intraesophageal inoculation of 1,000 *Giardia* cysts given via a blunt-ended feeding needle. The strain of *G. muris* used in this work was originally isolated from an infected golden hamster, and cysts of this strain were generously supplied by Dr. Frank W. Schaefer, Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio (Schaefer et al., 1984). To monitor the time-course of *G. muris* infection, fecal specimens were collected for 2-hr periods at various times during the first 5 wk after infection of mice with *Giardia* cysts and the number of fecal cysts was determined, using an established method (Roberts-Thomson et al., 1976). Briefly, mouse fecal specimens were broken up in water, and the resulting aqueous suspensions were centrifuged over 1 M sucrose. *Giardia muris* cysts were then aspirated from the interface above the sucrose layer and counted by microscopic examination in a hemacytometer chamber. Mice of 2 different ages were studied, viz. sexually immature and sexually mature animals (aged 22 days and 53 days, respectively, at the time of intraesophageal administration of *G. muris* cysts).

Within each age-group, the time-course of *G. muris* infection was similar in male and female mice, as judged by fecal *Giardia* cyst numbers (Table I). In the case of sexually mature mice, larger mean numbers of cysts were excreted by males than by females at several time-points, but the differences in cyst output by males vs. females were not statistically significant (Table I). As shown in the table, older mice excreted substantial numbers of cysts ($>10^4$/2 hr) over a longer period than did younger mice. Although the difference in cyst excretion by older vs. younger mice is noteworthy, the biological significance, if any, of this difference is unknown. In both age-groups of mice, the decreasing output of *Giardia*

TABLE I. *Numbers of* Giardia muris *cysts in 2-hr fecal specimens collected from male and female BALB/c mice, at various times after the start of* G. muris *infection.*

Number of days after start of infection*	*G. muris* cyst count, $\times 10^{-4}$†			
	Experiment 1‡		Experiment 2§	
	Male	Female	Male	Female
4			153 ± 36	63 ± 27
6	0	0		
7			45 ± 12	28 ± 6
8	<1	0		
11			46 ± 12	27 ± 6
12	25 ± 7	22 ± 8		
13			16 ± 4	11 ± 3
14	23 ± 6	26 ± 7		
16	<1	0		
19			6 ± 2	5 ± 2
20	<1	0		
21			5 ± 2	17 ± 7
23	0	0		
27	0	<1	0	<1
30	<1	0		
32			<1	<1

* Mice were infected with *G. muris* cysts on day 0.

† Mean values ± standard errors.

‡ Sexually immature mice; n = 12 males and 12 females (except for day 14, where data for 11 female mice are shown).

§ Sexually mature mice; n = 10 males and 9 females. Statistical analysis of the data in experiment 2 showed no significant differences between the numbers of cysts excreted by male vs. female mice ($P > 0.05$ by Student's *t*-test, on days 4, 7, 11, and 13 after start of infection).

cysts at the later time-points studied suggests that the infection was being cleared immunologically (Brett and Cox, 1982).

Although the precise mechanism by which immunocompetent mice clear *G. muris* infection is uncertain, there is considerable evidence that *Giardia*-specific antibody (rather than cell-mediated immunity) is responsible for the clearance of this infection from the mouse intestine (Heyworth, 1986; Heyworth et al., 1987). The present work shows that the time-course of an initial *G. muris* infection is essentially identical in male and female immunocompetent BALB/c mice and suggests that there is no sex-related difference in the production of *Giardia*-specific antibody by these animals, during an initial infection with the parasite. Whether there is any sex-related difference in the time-course of cyst excretion in mice reinfected with *G. muris*, after clearance of a primary infection (Brett and Cox, 1982), is a question that was not addressed in the present study.

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The Effects of Bile on the Locomotory Cycle of *Fasciola hepatica*

M. V. K. Sukhdeo, S. Keith, and D. F. Mettrick, Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

ABSTRACT: The rate of production of locomotory cycles in juvenile *Fasciola hepatica* was significantly decreased following treatment with conjugated chenodeoxycholic acids and significantly increased following treatment with dehydrocholic acid or whole bovine bile. Deoxycholic acid caused death and lysis of the parasites in less than 30 min. These results suggest that bile components act as a specific stimulus for a fixed action pattern (the locomotory cycle) in this parasite.

Bile is of critical importance in the biology of many gastrointestinal nematodes, cestodes, and trematodes, and, in many of these parasites, bile increases the activity of the infective stages (Lackie, 1975). The mode of action of bile is unclear but is believed to be dependent on the physical and chemical properties that produce a nonspecific stimulation of muscle activity (Dixon, 1966). This hypothesis is supported by the finding that the effects of bile on cestode activation can be mimicked by common detergents (Campbell, 1963). However, it has been recently argued that bile may be a receptor-mediated

TABLE I. *The behavioral response of juvenile* Fasciola hepatica *to various treatments.**

Treatment	Locomotory cycles	Distance travelled (mm)	Angular velocity (degrees/5 cycles)	Aborted cycles
Deoxycholic	(151.8 ± 84.9)†	(15.6 ± 7.0)	(52.4 ± 6.3)	(133.1 ± 128.7)
Glycochenodeoxycholic	25.7 ± 8.4^{a}‡	3.2 ± 0.8^{a}	30.8 ± 3.7^{a}	11.8 ± 2.7^{a}
Taurochenodeoxycholic	27.3 ± 20.8^{a}	4.8 ± 3.1^{a}	34.9 ± 12.4^{ab}	10.4 ± 4.8^{a}
Taurodeoxycholic	50.5 ± 37.2^{ab}	3.9 ± 1.9^{a}	69.7 ± 16.1^{b}	32.7 ± 23.1^{a}
Taurocholic	51.7 ± 27.5^{ab}	6.0 ± 2.8^{a}	58.5 ± 5.3^{ab}	30.1 ± 6.1^{a}
Glycocholic	59.1 ± 27.4^{abc}	7.1 ± 1.9^{a}	50.8 ± 0.2^{ab}	24.1 ± 4.7^{a}
Ringers (control)	80.1 ± 40.1^{bc}	9.4 ± 3.5^{ab}	50.4 ± 8.4^{ab}	23.7 ± 4.9^{a}
Glycodeoxycholic	93.3 + 44.7^{bc}	15.6 ± 7.8^{bc}	59.5 ± 7.5^{b}	32.0 ± 9.7^{a}
Cholic	103.1 ± 43.4^{c}	13.2 ± 5.1^{ab}	51.6 ± 6.5^{ab}	26.4 ± 4.0^{a}
Dehydrocholic	182.4 ± 47.4^{c}	23.9 ± 5.5^{c}	61.0 ± 6.4^{b}	71.1 ± 18.5^{b}
Bile (1%)	276.7 ± 97.6^{d}	23.7 ± 6.1^{c}	58.7 ± 12.7^{ab}	22.8 ± 8.4^{a}

* Observation period = 20 min, n = 7, $\bar{x}$ ± SE, for control and experimental groups.

† Bracketed values are calculated estimates of worm behavior based on observations before death. These values were not included in the statistical analysis (see text for further explanation).

‡ For each column, all values with identical superscripts are not significantly different from each other ($P < 0.05$).

stimulus (Sukhdeo and Mettrick, 1986, 1987). In the present study, the effects of bile and its components on a fixed action pattern of *F. hepatica* were examined. A fixed action pattern is a complex motor act, involving a specific temporal sequence of component acts, that is generated internally or elicited by a sensory stimulus (Shepherd, 1983). Fixed action patterns are not typically responsive to nonspecific stimulation (Kandel, 1976). The locomotory cycle in *F. hepatica,* a smooth sequence of whole-body waves coordinated with oral and ventral sucker activity that results in the characteristic "inchworm" locomotion, is a fixed action pattern (Sukhdeo and Mettrick, 1986).

Metacercariae of *F. hepatica* (Baldwin Enterprises, Monmouth, Oregon) were excysted artificially (Dixon, 1966; Smith and Clegg, 1981) and washed 5 times in buffered Hedon-Fleig ringers (pH 7.9). Worm activity was recorded using a video recorder (Panasonic NV 9240xD) with a time–date generator (Panasonic WJ-810) and a high-resolution low-light intensity video camera (Panasonic WV-1850) mounted on a compound microscope equipped with dark-field lighting. Experiments were done in Sykes-Moore culture chambers (Bellco Glass Inc., New Jersey) kept at 39 ± 0.5 C. The floor of the chamber was layered with 400 μl 0.25% agarose to provide purchase for worm suckers. Worms were placed in 500 μl test solution, and, after an initial 10-min acclimatization period (to allow the temperature in the chamber to equilibrate), worm behavior was continually recorded for 20 min. All test solutions were made in H-F ringers at a concentration of 1 mM, with the exception of bile (1.0%). Reagents were purchased from Sigma Chemical Co., St. Louis. Video analysis yielded: (1) locomotory cycles—identified by backward-moving waves starting at the anterior and terminating at the posterior end; (2) aborted locomotory cycles—as in (1) but backward waves terminated centrally; (3) distance travelled—path of ventral sucker traced onto acetate sheets; and (4) angular velocity—the absolute angular change per 5 locomotory cycles. Data were analyzed by 1-way analysis of variance and Duncan's multiple range tests. Significance was set at the 5% level.

When compared to ringer controls, 1% bovine bile and dehydrocholic acid significantly increased the production of locomotory cycles, whereas taurine- and glycine-conjugated chenodeoxycholic acids significantly decreased the production of locomotory cycles (Table I). Distance travelled was correlated with the number of locomotory cycles. There were no significant changes in angular velocity and an increase in the number of aborted locomotory cycles was only seen after treatment with dehydrocholic acid. Treatment with deoxycholic acid provoked lysis and death of all worms before the end of the experimental period (mean recording time = 5.1 ± 2.8 min). The bracketed values in Table I represent calculated estimates of worm behavior in deoxycholic acid based on observations before death; these values were not included in the statistical analysis.

These results demonstrate that bile, and specific components of bile, can alter the production of fixed action patterns in worm locomotion. Therefore, the effects on worm activity cannot

be attributed to nonspecific stimulation of muscle activity. The worm response suggests a receptor-mediated sensitivity that can distinguish among bile acids. Bile is composed of water, mucin, protein, bile pigments, bile salts (conjugated and unconjugated), phospholipids, neutral fats, and various organic ions (Haslewood, 1978). These constituents vary with diet, pH, and host microflora (Smyth and Haslewood, 1963; Haslewood, 1978). In addition, the types and relative quantities of bile acids differ among hosts, for example, ox bile may contain taurine and glycine conjugates of cholic acid, chenodeoxycholic acid, lithocholic acid, sapocholic acid, and stercholic acid (Haslewood, 1967). Not all bile acids were tested in this study, but the strong response stimulated by whole bile suggests a more complex picture in the activation of parasites than a response to a single bile component.

It has been argued that the action of bile is a factor that determines host specificity in *Echinococcus granulosus* (Smyth and Haslewood, 1963). This hypothesis was based on the finding that a primary component of herbivore bile, deoxycholic acid, which is not usually found in carnivores, causes lysis and death of the carnivore parasite. In the present study, deoxycholic acid at a much lower concentration (1 mM) than used by Smyth and Haslewood (4 mM) also provoked lysis and death in *F. hepatica*. Because *F. hepatica* is a parasite of both herbivores and carnivores, these results suggest that the relationship between bile and host specificity may not be as clear-cut as was suggested by Smyth and Haslewood.

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Toxoplasma gondii Maintenance in Tissue Culture: A New Efficient Method for Culturing RH Tachyzoites

Fernando R. Noriega and William E. Hauser, Jr., Evans Memorial Department for Clinical Research and Section of Infectious Diseases, Department of Medicine, Boston University Medical Center, Boston, Massachusetts 02118

ABSTRACT: We describe here a new tissue culture method for prolonged laboratory maintenance of tachyzoites of the highly virulent RH strain of *Toxoplasma gondii*. Using a rapidly proliferating murine tumor cell line (YAC-1), the method described is easy to perform and is as or more efficient (both in terms of yield and cost) than other traditional methods for maintenance of the parasite. Furthermore, upon prolonged maintenance (>160 days) in YAC-1 tissue culture, the pathogenicity of the parasite, as well as its capacity to elicit an immune response, are comparable to that of organisms maintained in mice. We conclude therefore, that the method described herein is a suitable alternative to the traditional method of maintenance of virulent RH strain *T. gondii* tachyzoites.

Toxoplasma gondii is an obligate intracellular parasite that traditionally has been passaged in animals (primarily the mouse) for its continuous laboratory maintenance and availability. Animal

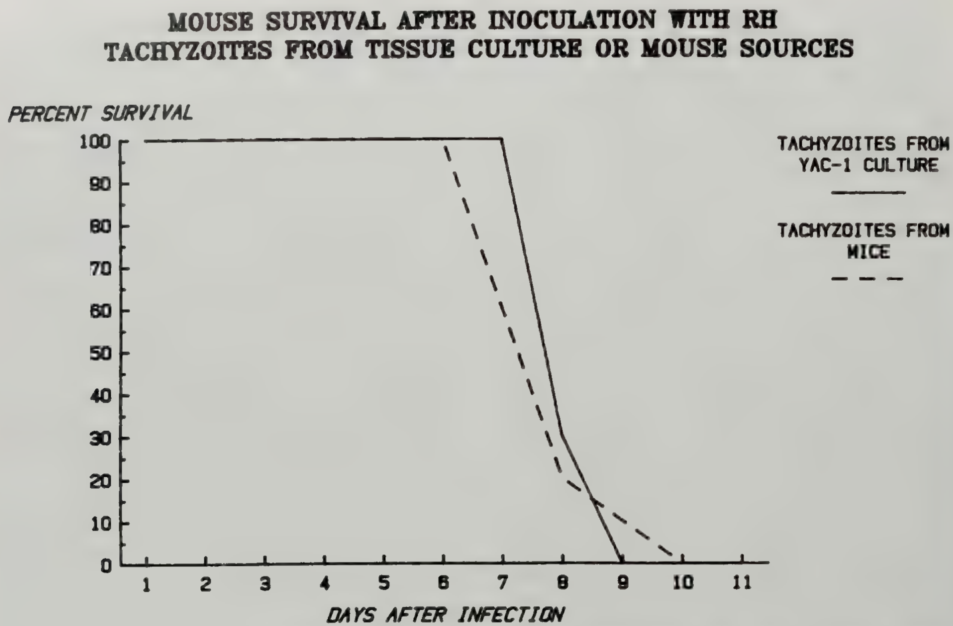

FIGURE 1. Groups of 10 mice were inoculated ip with 1×10^3 RH tachyzoites from YAC-1 culture or mouse sources. Mortality was assessed on a daily basis. Log rank statistics showed no statistical difference between drawn survival curves.

maintenance of the parasite, however, can become problematic, especially in terms of cost. As such, a variety of adherent, normal host cell lines have been used for routine maintenance of toxoplasma tachyzoites in tissue culture (Hogan et al., 1961; Lund et al., 1963; Paine and Meyer, 1969; Pfefferkorn and Pfefferkorn, 1976). These methods, nevertheless, have not supplanted the use of animal passage of the organism, especially for the production of large numbers of tachyzoites. Furthermore, little information is currently available regarding the potential utility of rapidly proliferating nonadherent tumor cell lines for maintenance of tachyzoites *in vitro*.

The purpose of this study, therefore, was to determine the usefulness of a nonadherent tumor cell line, the murine lymphoma YAC-1, for the tissue culture maintenance of virulent RH strain toxoplasma tachyzoites. In addition, studies were performed to assess the capacity of tachyzoites maintained in mice and in YAC-1 culture to cause lethal infection in mice and to infect normal murine macrophage monolayers. Studies were also performed to assess the effect of prolonged YAC-1 culture maintenance of the RH tachyzoites on their capacity to nonspecifically enhance murine NK cell tumoricidal activity and to elicit a *T. gondii*-specific antibody response in normal mice.

YAC-1 is a T-cell lymphoma induced by Moloney leukemia virus in A/SN mice. YAC-1 was originally provided to us by Dr. Howard Holden of the National Center Institute. This tumor cell line has been maintained continuously in our laboratory in stationary suspension culture (Hauser et al., 1982) in RPMI medium with 10% horse serum (RPMI-HS). RH tachyzoites are routinely maintained continuously in our laboratory in outbred CD-1 female mice (Charles River Laboratories, Kingston, New York) by serial intraperitoneal (ip) inoculation every 2 days. Purified RH tachyzoites prepared initially from mice (Hauser et al., 1982) were suspended in RPMI-HS and added to fresh YAC-1 cell cultures to result in a final concentration of 4×10^5 tachyzoites/ml and 2×10^5 YAC-1/ml in either 25-cm^2 (5-ml) or 75-cm^2 (15-ml) disposable tissue culture flasks (Corning, Corning, New York). Cultures were then incubated at 37 C in a humidified atmosphere of 5% CO_2. Intracellular infec-

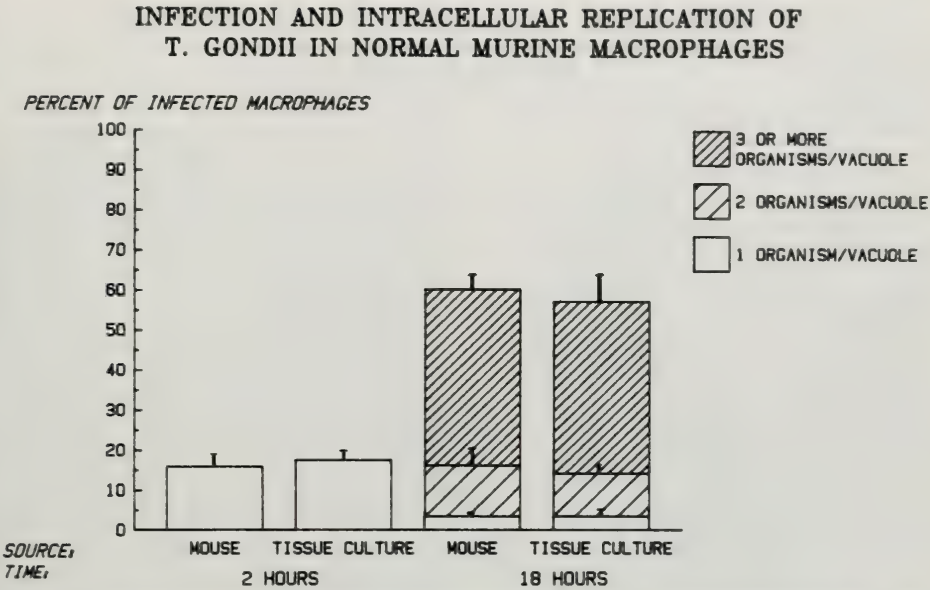

FIGURE 2. Normal murine macrophage monolayers were infected with RH tachyzoites from mouse or YAC-1 culture sources. Columns represent mean ± SD of the percent of infected macrophages at each time point as assessed by light microscopy. By chi-square analysis, no statistically significant differences were found in the percent of infected macrophages at 2 hr (columns on the left) or in the percent of infected macrophages with intracellular replication (organisms per vacuole) at 18 hr after infection (columns on the right).

tion and parasite replication was confirmed by phase-contrast inverted microscopy. Forty-eight hours later the majority of the lymphoma cells demonstrated infection with multiple intracellular tachyzoites. At this point, the infected cultures were maintained by 1 of the following methods: *Method A:* Blind transfers were made by mixing 2–20% of the total volumes of 2-day-infected YAC-1 cultures with the respective proportions of fresh YAC-1 cultures prepared as described above. *Method B:* Infected YAC-1 cells were passaged every 2 days as in protocol A, except that the volumes of infected cultures to be transferred were adjusted to concentrations ranging from 2 to 4 × 10^5 tachyzoites/ml of fresh tissue culture (i.e., 1:1 to 2:1 initial parasite: YAC-1 cell ratio).

To assess parasite yields for each one of the *in vitro* culture methods described above, 1-ml aliquots of each of the infected cultures were forced through a small gauge needle to release intracellular parasites, which were then quantitated by counting in a hemocytometer with trypan blue. Tachyzoite viability was always >95%. The protocols that allowed for the best parasite yields (average ± SE of tachyzoites/ml of infected tissue culture) were obtained by transferring 10% of the total volume of a 2-day-infected culture to a fresh culture of the same volume (yield = 7.97 ± 1.27 × 10^6 tachyzoites/ml), or by transferring the volume of an infected culture that was sufficient to result in a concentration of 3.4 × 10^5 parasites/ml of the fresh culture (yield = 7.09 ± 0.7 × 10^6 tachyzoites/ml). Lower inoculums produced lower parasite yields, whereas higher inoculums produced yields with a greater variance, making parasite production unreliable (data not shown).

The cost to produce 1 × 10^8 RH tachyzoites in YAC-1 culture was computed and compared against cost to produce the same amount of parasites in mice or in murine fibroblast (L929) cultures, the latter inoculated and maintained in a manner exactly similar to that described for the YAC-1 cultures. Considering the cost of materials, the YAC-1 tissue culture and animal maintenance of the parasite proved to be the most economic ($1.99 and $2.24, respectively), whereas fibroblast tissue culture maintenance of the parasite was more expensive ($5.05). The

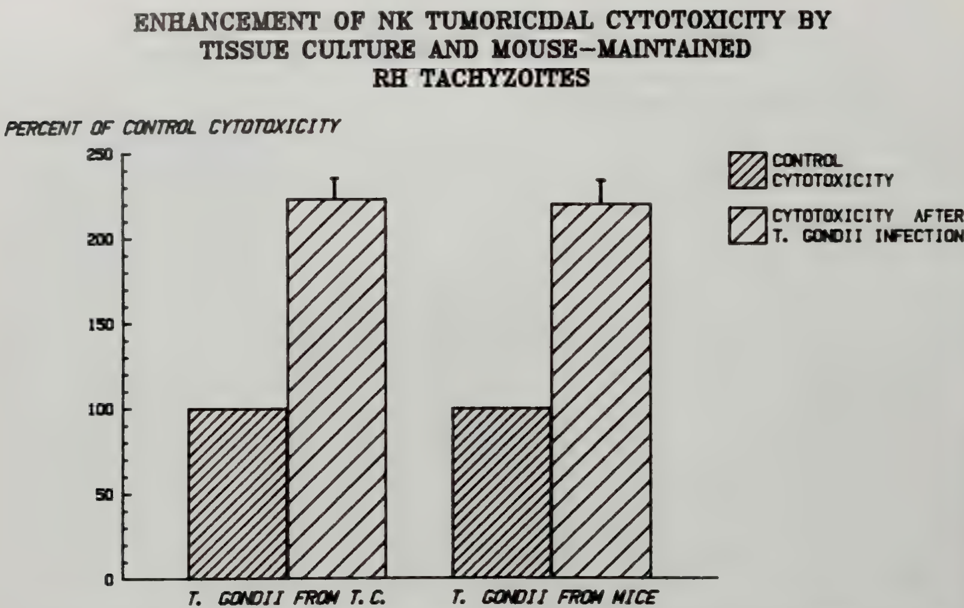

FIGURE 3. Normal mice were ip inoculated with 5 × 10^3 RH tachyzoites from YAC-1 culture or mouse sources. Three days thereafter, spleen NK cells were purified and their tumoricidal cytotoxicity determined in a standard chromium[51] release assay. Enhancement of spleen NK cytotoxicity was evaluated by the percent of control cytotoxicity ([cpm of Tg-stimulated cells/cpm of control cells] × 100). Columns in the figure represent means ± SD of percent control cytotoxicity. No statistically significant differences between groups were found by *t*-test.

elevated cost of parasite production in fibroblast cell culture was caused by the low yield of tachyzoites per flask (avg. 1.6 × 10^7/25-cm^2 flask for infected L929 culture compared with avg. 3.5 × 10^7/25-cm^2 flask for infected YAC-1 culture), necessitating the use of more (or larger-sized) tissue culture flasks and medium to produce the same amount of parasites as that produced in YAC-1 cultures. It should be noted that the above costs do not reflect the personnel costs incurred to maintain uninfected mice or tissue cultures. However, were such costs to be factored, the overall cost of animal maintenance to produce 1 × 10^8 tachyzoites would be increased to $9.80, and the cost to produce the same amount of parasites by YAC-1 lymphoma culture would be $5.79, and by L929 fibroblast culture $12.65 (cost computed using local hourly wage scales).

Despite the aforementioned data suggesting the apparent utility of YAC-1 tumor cells for tachyzoite maintenance, we nevertheless were concerned about any potential adverse effects of prolonged YAC-1 culture on parasite virulence and/or immunogenicity. Accordingly, a series of experiments were performed with parasites maintained in YAC-1 culture for over 160 days, and with parasites maintained only in mice in order to compare their capacity to cause lethal infection in normal mice, to infect and multiply within normal murine macrophages (MO) (Remington et al., 1972), to nonspecifically enhance spleen NK cell tumoricidal cytotoxicity (Hauser et al., 1982), and to elicit a *T. gondii*-specific antibody response in normal mice (Araujo and Remington, 1984). For each such study, parasites were purified as previously described (Hauser et al., 1982) to result in tachyzoite suspensions containing <0.5% host cells regardless of host source.

As shown in Figure 1, no apparent loss in the ability to cause lethal infection was observed as there were no significant differences between the survival curves of mice infected with tachyzoites that had been maintained *in vitro* compared to animals infected with parasites that had been maintained *in vivo.* Furthermore, as shown in Figure 2, no significant differences were observed in the ability of parasites to infect and replicate within normal nonelicited peritoneal MO *in tro,* as the percentage of MO infected, or the

divisions per vacuole within infected MO, was essentially equivalent at any time point regardless of the source of parasites used.

In terms of subsequent immunogenicity *in vivo*, as shown in Figure 3, augmentation of spleen NK cell cytotoxicity during acute *T. gondii* infection of mice was the same regardless of whether the tachyzoites used to produce infection were originally maintained in tissue culture or in animals, and in each instance, the level of augmentation observed was significantly increased over that of the respective control groups. Furthermore, when crude tachyzoite antigen prepared from sonicates of organisms was inoculated ip initially and then again 2 wk later, similar antibody responses were observed, as dye-test antibody titers were identical (1:512) regardless of whether mice had been immunized with sonicates prepared from tachyzoites originally maintained in mice or in YAC-1 tissue culture.

In conclusion, the present study describes a new method for tissue culture maintenance of virulent RH strain *T. gondii* tachyzoites using the nonadherent murine lymphoma cell line, YAC-1. On the basis of the results described herein, tachyzoite production in terms of yield and cost is at least equivalent to or better than the more traditional method of parasite maintenance in mice as well as an alternative method of maintenance in an adherent fibroblast cell line. Moreover, maintenance of tachyzoites in YAC-1 culture, even over prolonged periods of time, does not appear to have any untoward effects on the parasite's subsequent virulence and/or immunogenicity in permissive host cells or in normal animals.

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A New Annelid Intermediate Host (*Lanassa nordenskioeldi* Malmgren, 1866) (Polychaeta: Terebellidae) for *Aporocotyle* sp. and a New Final Host Family (Pisces: Bothidae) for *Aporocotyle simplex* Odhner, 1900 (Digenea: Sanguinicolidae)

Marianne Køie* and Mary E. Petersen†, *Marine Biological Laboratory, DK-3000 Helsingør, Denmark and †Zoological Museum, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark

ABSTRACT: *Aporocotyle* sp. (possibly *A. simplex*) was found in specimens of the polychaete *Lanassa nordenskioeldi*. *Aporocotyle simplex* is a blood fluke of fish and the only known digenean with a polychaete as the sole intermediate host.

Although metacercariae are not uncommon in Polychaeta, specimens infected with sporocysts or rediae are rarely collected. To date only 4 species of polychaetes (belonging to the families Ampharetidae, Terebellidae, and Serpulidae), each infected with a different species of sanguinicolid cercaria (Trematoda), have been found, suggesting that blood flukes that use polychaetes as intermediate hosts are species specific with

regard to their hosts (Køie, 1982, Ophelia **21**: 115–145).

Recently, our attention was drawn to a drawing and description of a fifth species of polychaete infected with larval digeneans. Wesenberg-Lund (1951, The Zoology of Iceland **2**(19): 1–182) found a specimen of "*Lanassa nordenskiöldi* (?)" from Seyðistörður, from a depth of 80 m, eastern Iceland, with the dorsal side distended by a large number of sausage-shaped, slightly curved bodies, each about 1 mm long (Fig. 1). Wesenberg-Lund (1951, p. 117) suggested these bodies to be "sporocysts containing a second generation of sporocysts of some trematode or other."

Reexamination of the infected polychaete, which is deposited in the Zoological Museum, University of Copenhagen (ZMUC), and whose identity was questioned by Wesenberg-Lund (1951, loc. cit.), confirmed the specimen to be *Lanassa nordenskioeldi* Malmgren, 1866 (Terebellidae), recently redescribed by Holthe (1986, Marine Invertebrates of Scandinavia **7**: 1–192). The species does not appear to be common and is only known from a few records; only 3 other samples of *L. nordenskioeldi* (reported by Wesenberg-Lund, 1953, Meddelelser om Grønland **122**(3): 1–169, 27 charts), all from the east coast of Greenland between 70°N and 75°N, are in the collections of the ZMUC, and none of these contain specimens with parasites. The other specimen of *L. nordenskioeldi* reported from Iceland by Wesenberg-Lund (1951, loc. cit., p. 112) was apparently not seen by her. It was reexamined by one of us (M.E.P.) and found to be *Melinna cristata* (M. Sars, 1851) (Ampharetidae); it contains no parasites.

The sausage-shaped bodies referred to by Wesenberg-Lund as sporocysts proved to be rediae filled with fully developed cercariae (Fig. 2). The rediae have a well-developed pharynx and an intestinal caecum. No daughter rediae (or sporocysts) were found. Examination of the rediae and cercariae using the light microscope and the stereoscan electron microscope revealed that they are morphologically indistinguishable from those of *Aporocotyle simplex* Odhner, 1900, a fish blood fluke and the only known digenean with a polychaete as the sole intermediate host. It is also the only marine sanguinicolid for which the life cycle has been worked out experimentally (Køie, 1982, loc. cit.; see table 2 in that paper for a comparison of the 9 then-known cercariae of marine sanguinicolids). The furcocercous cercariae (Fig. 3)

FIGURE 1. *Lanassa nordenskioeldi* from Iceland infected with rediae of *Aporocotyle* sp. At left, anterior end of polychaete in right dorsolateral view, showing swollen area with rediae ("sporocysts" of Wesenberg-Lund). At right, a single redia ("sporocyst," upside down in figure). Reproduced from the original drawing by Poul H. Winther for Wesenberg-Lund (1951, loc. cit., p. 116) with permission from The Zoology of Iceland.

develop in rediae in *Artacama proboscidea* Malmgren, 1866 (Terebellidae), and upon release penetrate the skin of pleuronectid fishes. After a stay in the lymphatic system of the fish, the digeneans mature in the branchial vessels and the heart (Køie, 1982, loc. cit.).

Two species of *Aporocotyle*, *A. simplex* and *A. spinosicanalis* Williams, 1958, have been recorded in the northern Atlantic Ocean and adjacent seas (Køie, 1982, loc. cit.). *Merluccius merluccius* (L.), present along the west coast of Europe, is the only known final host for *A. spinosicanalis* and there is little or no overlap between the distributions of this fish and that of *L. nordenskioeldi*. It thus seems unlikely that the digenean in *L. nordenskioeldi* is *A. spinosicanalis*. The distribution of *L. nordenskioeldi* is within the range of that of *A. simplex* even though

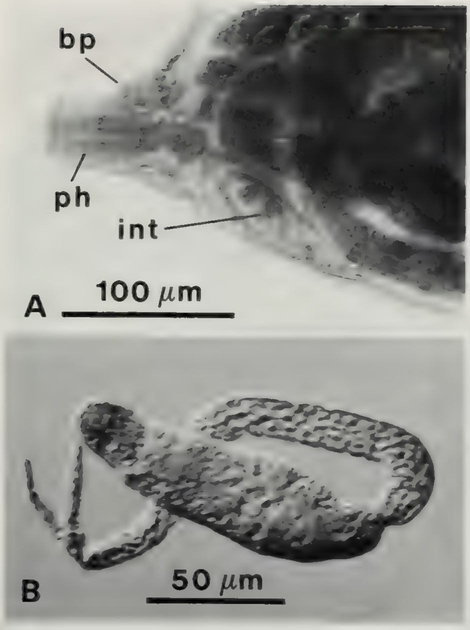

FIGURE 2. *Aporocotyle* sp. from specimen of *Lanassa nordenskioeldi* shown in Figure 1 (specimen presently stored in 80% ethyl alcohol, original fixation unknown). **A.** Anterior end of redia, postfixed with osmium tetroxide and mounted in Depex, showing pharynx (ph), intestinal caecum (int), location of birth pore (bp), and cercariae. **B.** Cercaria removed from redia and cleared in lactophenol.

FIGURE 3. Cercaria of *Aporocotyle simplex* from *Artacama proboscidea* from the Øresund. Drawn from living specimen, slightly flattened, with tail somewhat contracted. Reprinted from Køie (1982, loc. cit., p. 119) with permission from Ophelia.

A. simplex has not yet been recorded from east of Iceland. Thus, although we cannot prove that the digenean in *L. nordenskioeldi* is *A. simplex* on the basis of the present material, we cannot find any morphological or geographical criteria for separating it from this species, and a final decision as to its identity must await the study of additional material.

Aporocotyle simplex has previously been reported only from members of the fish family Pleuronectidae. However, examination of 10 specimens (30–60 cm long) of *Scophthalmus maximum* (L.) (Bothidae) from the northern Øresund, from a depth of 25 m, Denmark (August 1987) revealed a 100% infection with *A. simplex* (ca. 20–50 worms per fish), whereas none of 7 specimens of *S. rhombus* (L.) taken in the same dredge hauls were infected with this species. Also 40 specimens of *Microstomus kitt* (Walbaum) (Pleuronectidae) living at the same depth in the Øresund and close to the infected polychaete host (*Artacama proboscidea*) were uninfected. The skin of *S. maximus* is rough, whereas that of *S. rhombus* and *M. kitt* is both smooth and slimy; it is therefore suggested that some structural or chemical aspect of the skin of th latter 2 species may hinder attachment and etration by cercariae of *A. simplex*.

Rediae and cercariae of *Aporocotyle* sp. from

the infected specimen of *Lanassa nordenskioeldi* from Iceland, and of *A. simplex* from *Artacama proboscidea* from the Øresund have been deposited in the Zoological Museum, University of Copenhagen, and in the U.S. National Museum Helminthological Collection.

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Transmission of the OS Strain of *Plasmodium inui* to *Saimiri sciureus boliviensis* and *Aotus azarae boliviensis* Monkeys by *Anopheles dirus* Mosquitoes

William E. Collins, Jimmie C. Skinner, Valery Filipski, Carla Wilson, J. Roger Broderson, and Peggy S. Stanfill, Division of Parasitic Diseases and Office of Scientific Services, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia 30333

ABSTRACT: Eight *Saimiri* and 7 *Aotus* monkeys were exposed to infection with the OS strain of *Plasmodium inui* via the bites of from 2 to 7 *Anopheles dirus* mosquitoes. All *Saimiri* monkeys developed high-level infections of from 152,000 to 500,000/mm³ after prepatent periods of from 14 to 17 days. Only 1 *Aotus* monkey developed a patent infection after a period of 28 days. Feeding on these animals failed to result in infection of *An. dirus* mosquitoes.

Plasmodium inui, a quartan-malaria parasite of macaques, is used as a model for the human malaria parasite, *P. malariae.* These parasites are characterized by an acute infection followed by long periods of chronic infection. The *P. inui shortti* subspecies (Eyles, 1963) has been maintained by us as the OS strain of *P. inui* (Coatney et al., 1971). This particular parasite has been grown *in vitro* (Nguyen-Dinh et al., 1980) and has been shown to be infectious to humans (Coatney et al., 1966). Previous studies had indicated that following inoculation of parasitized blood, the parasite would develop in *Aotus lemurinus griseimembra* (Collins et al., 1981) and in *Saimiri sciureus* from Guyana and Bolivia (Chin et al., 1983). A single transmission to a splenectomized *Saimiri* of Guyanan origin was obtained by the intravenous inoculation of infected salivary glands dissected from 4 *Anopheles freeborni* mosquitoes.

Saimiri and *Aotus* monkeys are of considerable interest for numerous malarial studies including chemotherapeutic as well as vaccine development. Presented here are the results of attempts to determine the susceptibility of *Saimiri* and *Aotus* to infection via the bites of mosquitoes infected with the OS strain of *P. inui.*

All animals were splenectomized prior to being challenged and had been previously used in various studies with *P. vivax, P. falciparum, P. malariae,* or *P. simium. Anopheles dirus* mosquitoes were infected by feeding on a *Macaca mulatta* monkey that had been infected with the OS strain by intravenous inoculation of parasitized blood. After 17 days of extrinsic incubation, mosquitoes were fed on the tranquilized *Saimiri* and *Aotus* using procedures previously reported (Collins et al., 1966). After engorgement, the mosquitoes were dissected and the salivary glands graded 1+ to 4+ based on the number of remaining sporozoites (1+ = 1–10 sporozoites, 2+ = 11–100 sporozoites, 3+ = 101–1,000 sporozoites, and 4+ = >1,000 sporozoites).

Eight splenectomized *Saimiri sciureus boliviensis,* all of which had previously been infected with *P. vivax,* received the bites of 2–5 heavily infected (4+) *An. dirus* (Table I). Patent parasitemias developed in all animals after prepatent periods of 14–17 days (mean of 15 days). Parasitemias rose rapidly and reached maximum levels of 152,000–508,000/mm³ after 13–20 days of patent parasitemia (mean of 16.9 days). Four animals were treated with chloroquine (45 mg base over a 3-day period) when the parasitemia reached 400,000/mm³. Thirty-nine days after exposure to infection, the remaining 4 monkeys were given a similar treatment with chloroquine.

TABLE I. *Prepatent periods and parasitemias in* Saimiri *and* Aotus *monkeys infected with the OS strain of* Plasmodium inui *via the bites of* Anopheles dirus *mosquitoes.*

Animal	Species	Previous malaria*	Mosquito bites	Prepatent period	Maximum parasitemia	
					Per mm³	Day†
SS-53	*S. sciureus boliviensis*	Pv	5	15 days	508,000	35
SS-54	*S. sciureus boliviensis*	Pv	5	15 days	152,000	28
SS-55	*S. sciureus boliviensis*	Pv	4	17 days	268,000	33
SS-57	*S. sciureus boliviensis*	Pv	3	15 days	500,000	30
SS-64	*S. sciureus boliviensis*	Pv	2	14 days	324,000	31
SS-66	*S. sciureus boliviensis*	Pv	4	15 days	404,000	35
SS-67	*S. sciureus boliviensis*	Pv	4	15 days	324,000	35
SS-70	*S. sciureus boliviensis*	Pv	5	14 days	428,000	28
AI-280	*A. azarae boliviensis*	Pm, Ps	3	28 days	384,000	40
AI-281	*A. azarae boliviensis*	Pm, Ps	6	No infection		
AI-299	*A. nancymai*	Pf, Ps	5	No infection		
AI-300	*A. nancymai*	Pf, Ps	4	No infection		
AI-552	*A. nancymai*	Pf, Pv	5	No infection		
AI-553	*A. nancymai*	Pf, Pv	7	No infection		
AI-554	*A. nancymai*	Pf, Pv	6	No infection		

* Pv = *Plasmodium vivax*; Pf = *P. falciparum*; Pm = *P. malariae*; Ps = *P. simium*.
† Days after infection.

Anopheles dirus were fed on each of these animals (100 total feedings) during periods when the levels of parasitemia were in excess of 10,000/mm³. Subsequent dissection indicated that no mosquito infections occurred.

Three to 7 heavily infected (4+) *An. dirus* were fed on 7 splenectomized *Aotus* monkeys (5 *A. nancymai*, karyotype I and 2 *A. azarae boliviensis*, karyotype VI). In 1 *A. azarae boliviensis* (AI-280) a patent parasitemia developed after 28 days. The maximum parasite count obtained on day 12 of patent parasitemia was 384,000/mm³. All animals were treated with chloroquine 39 days after exposure to infection.

It is apparent that splenectomized *Saimiri* from Bolivia are highly susceptible to infection via sporozoite inoculation and could well serve in causal prophylactic drug studies and possibly in sporozoite vaccine trials. Their inability to infect mosquitoes, in spite of high-level infections, indicates that they would be of little use for sporogonic studies. Because only 1 of the 7 splenectomized *Aotus* developed detectable parasitemia, these monkeys would obviously be less useful than the highly susceptible *Saimiri*. However, the *Aotus* used in this study had extensive experience with other species of *Plasmodium* prior to challenge with the *P. inui* parasites. Whether or not *Aotus* with no previous infections would be more susceptible remains to be shown. In humans, previous infection with *P. vivax* or *P. falciparum* has not been shown to prevent infection with *P. malariae*.

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An Alternative Method for the Culture of *Diplostomum spathaceum* (Trematoda) in Chick Embryos

S. W. B. Irwin and D. H. Saville, Department of Biology, University of Ulster at Jordanstown, Shore Road, Newtownabbey, Co. Antrim BT37 0QB, Northern Ireland

ABSTRACT: Metacercariae of *Diplostomum spathaceum* were maintained in the allantoic cavities of chick embryos for 15 days. Some embryos had 0.2 ml chicken serum added to the allantoic cavity each day. Although the level of development varied considerably, worms from embryos with added serum developed hind bodies that were substantially larger than those of parasites maintained without added serum. There was no evidence that any worm ingested blood, and only 1 individual, from the serum-augmented group, became ovigerous.

Free metacercariae of *D. spathaceum* are found in the eye lenses of freshwater fishes. Following ingestion by birds, the metacercariae develop into adult trematodes and during maturation a hind body develops that contains the reproductive structures. Recently, Leno and Holloway (1986) cultivated this trematode on the outer surface of the chorioallantois of chick embryos. This technique, originally developed by Fried (1962), has been successfully utilized for a number of trematode species. In the present study, metacercariae of *D. spathaceum* were inoculated through the chorioallantoic membrane into the allantoic cavity. A comparison was made between trematodes allowed to develop for 15 days in this environment and others maintained in the same environment to which 0.2 ml chicken serum was added each day.

Leno and Holloway (1986) observed egg production in *D. spathaceum* grown *in ovo* in 1 organism that developed an egg that gave rise to a miracidium. In our study we assessed development by measuring the area of the fore body and hind body of each fluke and expressing the proportion of the hind body as a percentage of the entire body surface.

The method adopted for inoculation of *D. spathaceum* into chick embryos was very similar to that described by Blaškovič and Styk (1967) for intraallantoic inoculation of viruses. Free metacercariae were dissected from the eye lenses of rainbow trout (*Salmo gairdneri*) obtained from a local fish farm and they were washed in Hanks' balanced salts containing 50 μg/ml streptomycin and 50 IU/ml penicillin. Seven-day-old fertile chick eggs were swabbed with 70% ethanol and each had a small incision made on the uppermost surface of the shell. The metacercariae were injected, by means of a hypodermic syringe, through the underlying shell membrane and chorioallantoic membrane into the allantoic cavity. Each incision was again swabbed before being sealed with Scotch tape. Half of the eggs had 0.2 ml chicken serum injected into the allantoic cavity each day, and after 7 days incubation at 41 C, the chick embryos were examined and the chorionic endoderm and allantoic cavity were searched for developing flukes. The parasites were transferred to a new batch of 7-day-old fertile eggs to allow growth and development to continue for another 8 days.

Following a total of 15 days of worm development, the second batch of chick embryos was examined and specimens of *D. spathaceum* were cooled at 4 C for 1 min to reduce their activity so that measurements could be made under a compound microscope. The lengths and widths of metacercariae taken directly from rainbow trout eyes were also recorded so that comparisons with cultured flukes could be made.

After the initial period of 7 days development, 20–30% of the *D. spathaceum* were recovered. This figure applied to organisms maintained with additional chicken serum and those without. Eight parasites were transferred from a serum-augmented environment to a second chick embryo that also had chicken serum added each day, and 6 (75%) were recovered after an additional 8 days. Sixteen flukes cultivated *in ovo* without chicken serum were transferred to a second chick embryo without daily addition of chicken serum, and 6 (37.5%) were recovered on completion of the experiment. Mean measurements of these organisms are listed in Table I.

Berntzen and Macy (1969) adopted the rationale that length multiplied by width equals a

TABLE I. *Measurements (in µm) of metacercariae and 15-day-old* Diplostomum spathaceum *maintained* in ovo *with and without additional chicken serum.*

		Fore body		Hind body	
		Length	Width	Length	Width
Metacercariae (n = 26)	$\bar{x}$	50.64	38.56	—	—
	Range	38.4–72.0	25.6–52.0		
D. spathaceum in ovo without additional chicken serum (n = 6)	$\bar{x}$	52.0	35.74	21.86	15.74
	Range	32.8–73.6	24.8–44.0	2.4–29.6	6.4–20.0
D. spathaceum in ovo with additional chicken serum (n = 6)	$\bar{x}$	65.34	48.0	38.0	22.4
	Range	27.2–88.0	29.6–72.0	0.0–68.8	0.0–36.0

worm's body area. When applied in this case, the figures show that the hind body represented 15.6% of the total body in those developed without chicken serum and 21.3% of the total body of those maintained in the chick allantoic cavity with additional chicken serum each day.

Although reproductive organs such as testes, ovaries, and vitellaria were visible in a number of 15-day-old specimens of *D. spathaceum,* there was no evidence that blood had been ingested. Only 1 individual was observed with an egg in the uterus. It had been maintained in the environment with added chicken serum and its hind body composed 39.2% of its total body area.

The results obtained in this investigation compare favorably with those of Leno and Holloway (1986). In both cases 1 specimen of *D. spathaceum* was obtained with 1 egg in the uterus. This demonstrates that the allantoic cavity with additional chicken serum is as suitable as the outside of the chorioallantois for the development of this parasite. A significantly higher proportion of the flukes was recovered in the present study. This might indicate that the organisms were confined to this relatively hospitable environment, whereas when placed on the chorioallantoic surface they may move to less favourable areas or be subjected to desiccation.

The fact that there was no evidence of blood residues in the intestinal ceca of *D. spathaceum* in this study contrasts with *Himasthla quissetensis* (Fried and Groman, 1985), *Echinostoma revolutum* (Fried and Pentz, 1983), and *D. spathaceum* (Leno and Holloway, 1986), which, when grown on the surface of the chorioallantoic membrane, had hematin-like material in their ceca. Borysko and Bang (1953) stated that the chorionic outer layer of the chick chorioallantois is penetrated by blood vessels that form an extensive anastomosing capillary plexus on the outer surface, whereas the allantoic membrane on the inside is composed of a layer of epithelial cells. It is therefore not surprising that worms grown in the allantoic cavity are less likely to gain access to blood capillaries and perhaps it is this that renders chicken serum necessary to sustain the level of maturation observed by Leno and Holloway (1986) in *D. spathaceum* maintained on the outer surface of the chorioallantois.

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A Possible Instance of Autoinfection in a Pharyngodonid (Oxyurida) Parasite of *Amphisbaena alba* from Venezuela

Martin L. Adamson, Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada V6T 2A9

ABSTRACT: Larvae similar to those found in infective eggs of members of the order Oxyurida were found apparently free in the uterus of a female oxyuridan (perhaps belonging to the genus *Alaeuris*) recovered from *Amphisbaena alba* from Venezuela. The worm may reproduce by autoinfection as do several other Oxyurida (*Gyrinicola* spp., *Tachygonetria vivipara*, and certain *Alaeuris* spp.) of amphibians and lizards.

The vast majority of zooparasitic nematodes are serial colonizers; parasitic females produce eggs or larvae that must pass to the external environment to complete the life cycle. Autoinfection, the ability to cycle within the host, occurs, however, in certain Oxyurida of the Pharyngodonidae.

Typically, members of the order Oxyurida are transmitted by thick-shelled eggs contaminating the host's environment. However, a few species from amphibians and lizards are known to produce 2 types of eggs: a thick-shelled variety that must pass to the external environment to complete the life cycle and a thin-shelled variety that hatches at or soon after deposition and gives rise to an endogenous cycle (autoinfection).

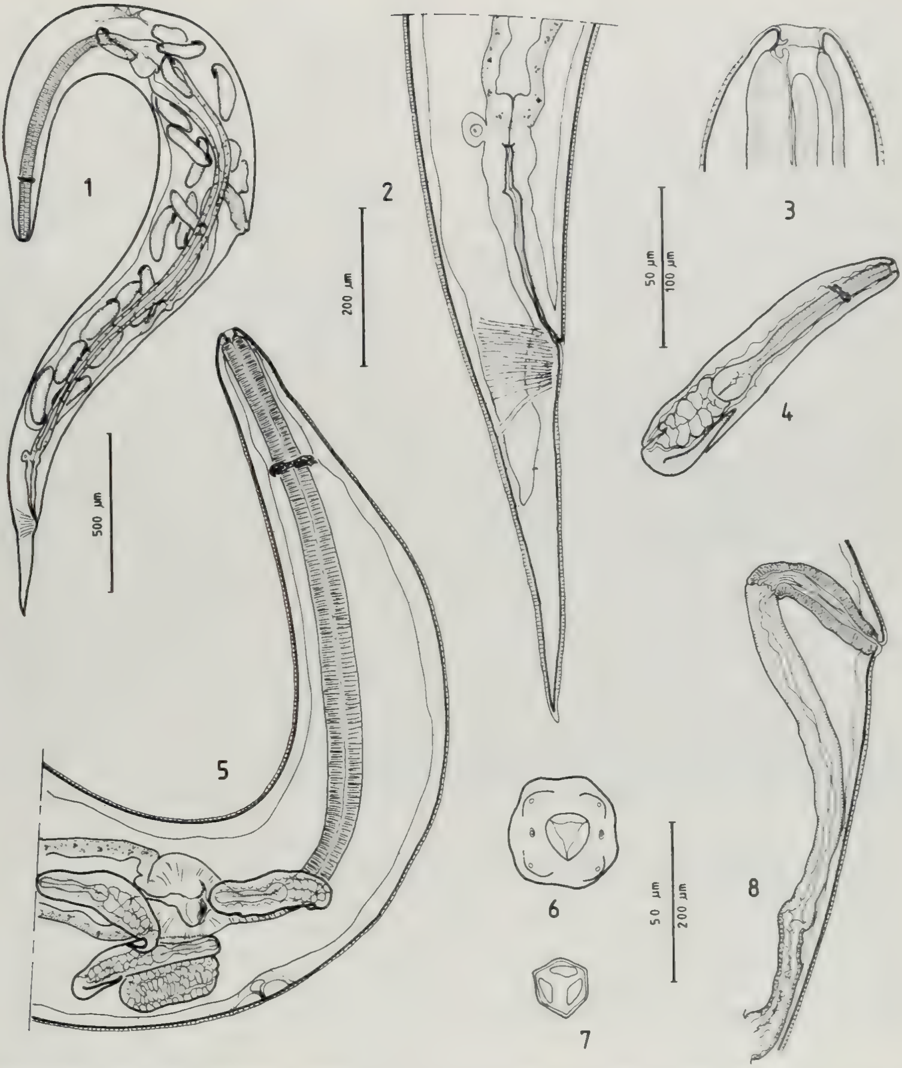
Recently, I obtained, on loan, nematode specimens from *Amphisbaena alba* (Amphisbaenidae) deposited in the Museum d'Histoire Naturelle in Paris (Laboratoire des Vers: 800F). In addition to a dozen Atractid specimens was a female nematode (Figs. 1–8) attributable to the family Pharyngodonidae, perhaps belonging to the genus *Alaeuris*. A remarkable feature of this worm is that larvae developing *in utero* are not surrounded by an egg shell but apparently lie free in the uterus. The only other Oxyurida in which an egg shell is lacking (or extremely reduced) are those that exhibit autoinfective cycles and the present material therefore seems to represent another such species.

The specimen, 3.02 mm long, is described as follows. Maximum width 322 μm near midbody. Buccal cavity 6.3 μm, oesophageal corpus 748 μm, and isthmus 100 μm long. Bulb 103 μm long and 113 μm wide. Nerve ring 187 μm, excretory pore 865 μm, and vulva 1.5 mm from anterior extremity. Vagina consisting of thick-walled portion nearest vulva 177 μm long, and posteriorly directed vagina uterina 645 μm long. Tail conical, 481 μm long.

Pharyngodonid genera are difficult to distinguish uniquely on the basis of female morphology. Nevertheless the present material is likely a member of *Alaeuris* or *Paralaeuris* because these are the only pharyngodonid genera from New World lizards (with the exception of *Ozolaimus*, distinguishable on the basis of oesophageal and cephalic morphology) in which the female has a conical tail. I have not given the specimen a species name because only a single female specimen was available for study.

Autoinfection is known in only 3 oxyuridan genera. Species of the genus *Gyrinicola* parasitize anuran larvae throughout the world and all have an autoinfective phase in their life cycle (Adamson, 1981a, 1981b). Such a phase also occurs in *Tachygonetria vivipara*, a parasite of *Uromastix acanthinurus* in North Africa, and in 2 species of *Alaeuris*, namely *A. vogelsangi* and *A. caudatus* parasitizing *Iguana iguana* in South America (Seurat, 1913; Petter, 1969; Adamson and Petter, 1983).

Unlike the situation in Atractidae (Cosmocercoidea; Ascaridida) where autoinfection occurs in each generation, autoinfective generations apparently alternate with dispersing generations in the above Oxyurida. This is accomplished in at least 2 ways. In *Gyrinicola*, females produce 2 types of eggs (a thin-shelled autoinfective type and a thick-shelled dispersing type) in separate horns of the reproductive tract. One egg type tends to predominate in a given female and there is an alternation of generations such that females that develop from thin-shelled eggs produce almost exclusively thick-shelled eggs, whereas those that develop from thick-shelled eggs produce predominantly thin-shelled eggs (Adamson, 1981c). In *Alaeuris, Tachygonetria*, and presumably the material described here, there are 2 types



FIGURES 1–8. Pharyngodonid (Oxyurida) specimen from *Amphisbaena alba* from Venezuela. **1.** Entire worm, lateral view. **2.** Caudal extremity, lateral view. **3.** Cephalic extremity, lateral view. **4.** Larvae from uterus. **5.** Oesophageal region. **6.** Apical view. **7.** Optical section through buccal capsule. **8.** Vagina. Scale bars: Figure 1 = 500 μm; Figures 2, 5, and 8 = 200 μm; Figures 3, 6, and 7 = 50 μm; Figure 4 = 100 μm.

of females. One type produces thick-shelled eggs that must pass out of the host to continue their development and another type produces thin-shelled autoinfective eggs. There is evidence to suggest that in these species also there is an alternation of generations (Adamson and Petter, 1983).

Thus, in all of the autoinfective oxuridans the basic pattern involves a generation of colonizing females that give rise to a second generation in the same host individual; worms of this second generation produce thick-shelled dispersing eggs. Members of the Oxyurida are haplodiploid, i.e., males develop from unfertilized eggs and are

haploid, whereas females develop from fertilized eggs and are diploid (Adamson, 1984). Adamson (1981c, 1984) and Adamson and Petter (1983) postulated that autoinfective cycles in the Oxyurida take advantage of the superior colonizing ability of the females. Thus, a single female can colonize a host if she can survive to mate and produce offspring with parthenogenetically produced sons. Male oxyuridans are typically smaller and mature more rapidly than their female counterparts (Adamson, 1981b) and there is evidence that mother–son matings occur in the wild (Adamson, 1981d).

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Growth of an *Acanthamoeba* Isolate on a Gram-negative Bacterium, Probably *Pasteurella haemolytica*

M. Maqueda*, E. Valdivia*, M. Martínez-Bueno*, M. L. Mascaró†, and C. Mascaró†, *Department of Microbiology, University of Granada, Spain; and †Department of Parasitology, University of Granada, Spain

ABSTRACT: A gram-negative bacterium, probably *Pasteurella haemolytica,* was found to support the growth of *Acanthamoeba* spp. This provides a useful means for initial isolation of *Acanthamoeba* spp. and for culturing these parasites to high cell densities.

The relationship between free-living amoebae and bacteria has been extensively studied by different authors, mainly by Singh (1941, Annals of Applied Biology **29:** 52–64; 1942, Annals of Applied Biology **30:** 316–325), Amonkar and Freitas (1964, Indian Journal of Microbiology **4:** 88–91), Chang (1960, Canadian Journal of Microbiology **4:** 397–405), and Wright et al. (1981, Journal of Microbiology **125:** 293–300). In the present study we found that a gram-negative bacterium supports growth of *Acanthamoeba* spp.

An epidemiological study related to a fatal human case of granulomatous amoebic encephalitis by *Acanthamoeba* sp. showed that the suspected source of contamination was water from a household filter used by the patient for nasal washings. From this filter we have isolated 2 strains of amoebae, *Hartmannella vermiformis* and an *Acanthamoeba* sp. strain. Also, 2 bacterial strains that grew with *Acanthamoeba* sp. in the initially established cultures (grown in Bacto-Difco-Agar dishes at 37 C) were isolated. In these cultures, brilliant and translucent accumulations bigger than bacterial colonies were observed. After microscopic observations and staining, these accumulations were shown to be mainly amoebae and gram-negative bacteria. The latter, when spread on plates containing bacteriological medium (Trypticase Soya Agar) gave rise to 2 different types of colonies.

Each strain of bacteria was isolated as Gr-A and Gr-B, respectively, and each tested indepen-

Table I. *Characterization of Gr-B strain compared with members of the family Pasteurellaceae.*

Characteristics	*Actino-bacillus*	*Haemo-philus*	*Pasteur-ella*	Gr-B
Requirement of factor V	*	+	–	–
Colonies sticky	+†	–	–	–
Growth on MacConkey agar	+	–	D	+
Test:				
Indole	D‡	D	D	–
Catalase	D	D	D	–
Oxidase	D	D	D	+
Urease	+§	D	D	+
Methyl red	–	D	–	–
Voges Proskauer	D		–	–
Ornithine decarboxylase	–	–§		
Orthonitrophenyl galactoside	+§	D	D	
Fermentation of:				
Glucose	+	+	+	+
Fructose	+	D	+	–
Xylose	+	D	D	+
Dulcitol	–	–	–§	+
Inositol	–	–§	D	+
Inulin	–	–§	–	

* Ninety percent or more negative strains.
† Ninety percent or more positive strains.
‡ Different reactions in different species.
§ Occasional strains may differ from this reaction.

dently to determine its effect on the growth and accumulation of the amoebae. Bacterium Gr-A, identified as *Pseudomonas maltophila,* did not modify the growth of the isolated *Acanthamoeba* sp. strain. The bacterium Gr-B, however, caused rapid growth and accumulation of amoebae.

Identification of Gr-B bacterium: The results obtained using the API-20E System (API System S.A., Appareils et Procédés d'Identification, France) and other assays (growth on MacConkey agar, catalase test, and glucose utilisation), pointed to a gram-negative facultatively anaerobic rod, probably belonging to the family Pasteurellaceae. The most relevant characteristics of this bacterium, according to recent bibliographic references (Noel et al. (eds.), Bergey's manual of determinative bacteriology, Vol. 1, Williams and Wilkins, Baltimore/London, p. 550), are: straight, rigid, coccoid to rod-shaped cells, with great pleomorphism, nonmotile, aerobic with varying degrees of microaerophilia, facultatively anaerobic, chemoorganotrophic, and with both respiratory and fermentative types of metabolism. Acid is produced by fermentation of glucose, other carbohydrates, sugar, alcohols, or glycosides. Nitrites are formed from nitrates. Oxidase and catalase are characteristically positive. The differential characteristics of the members of this family are shown in Table I. The genus *Haemophilus* was eliminated because bacterium Gr-B did not require Factor V. The close relationship of species of *Actinobacillus* and those of the *Pasteurella* group may pose difficulties in identification, and certainly presents taxonomic problems. Referring to the genus *Pasteurella,* the results obtained with the Gr-B strain (Table II) are not fully coincident with the 6 members of

Table II. *Differential characteristic of the species of the genus* Pasteurella *compared with Gr-B strain.*

Characteristics	*P. multocide*	*P. pneumotropica*	*P. haemolytica*	*P. ureae*	*P. aerogenes*	*P. gallinarum*	Gr-B
Hemolysis (β)	–*	–	+	–	–	–	–
Growth on MacConkey agar	–	–	+	–	+		+
Indole production	+†	+	–	–	-	–	–
Urease activity	·	+	–	+	+	–	+
Gas from carbohydrates		–	·		+	–	
Acid production from:							
Lactose							
Mannitol	v‡		+	+	–		+
Arabinose					+		+
Dulcitol			+	[-]			+
Fructose				+			
Glucose			+	+	+		+
Glycerol			+	[–]	+		+
Maltose			+		+		
Melibiose			+				+
Rafinose			+				+
Rhamnose			+		d§		+
Sorbose				[ ]	+		+
Xylose							+

* Ninety percent or more negative strains.
† Ninety percent or more positive strains.
‡ Strains unstable.
§ Eleven to 89% positive strains.

that group. However, there are many similarities between Gr-B strain and the following species: *P. ureae* responds to indole; ornithine decarboxylase and urease tests are the same, differing only in the orthonitrophenyl galactoside (ONPG) test and on MacConkey agar. *Pasteurella haemolytica* and *P. aerogenes* are the only species able to grow on MacConkey agar, as does Gr-B strain. However, this strain is neither haemolytic (sheep and human blood), nor aerogenic, which are characteristics that define the above mentioned species. The results obtained on the pattern of sugar fermentation (Table II) are closer to those exhibited by *P. haemolytica*. The latter, however, is urease-negative whereas Gr-B is positive. With regard to the genus *Actinobacillus*, one of its most outstanding features is the presence of coccal elements interspersed with bacillary cells, giving typical "morse code" forms. Those we have not detected. We also did not find sticky colony formation or the sugar fermentation pattern (especially with respect to dulcitol, sorbose, and fructose) that occurs in *Actinobacillus* sp. The capacity of the members of this genus to grow on MacConkey agar, which is considered by some authors as a sufficient criterion to distinguish it from *Pasteurella* sp., was not considered adequate because some species of the latter also grow on that agar medium.

According to all these considerations, we think that bacterium Gr-B most probably belongs to the species *Pasteurella haemolytica*. The lack of hemolytic capacity is not conclusive because this character can be lost after growth out of its natural habitat. We assume that extensive growth of the *Acanthamoeba* strain in the water filter may have been strongly favoured by the presence of the presumptive *P. haemolytica*. In order to verify if it occurs only with this species of *Acanthamoeba*, other species of this genus (*A. palestinensis* Reich, *A. castellanii* Neff, *A. culbertsoni* A1, *A. lugdunensis* SH 565, *A. lenticulata* PD2, and *A. griffini* S7) were grown on agar dishes with living cells of *P. haemolytica*. In all cases a notable increase in amoebic development was found. We recommend the use of this bacterium for the initial isolation of *Acanthamoeba* spp. upon agar dishes from fresh water, air, or pathological samples, due to its ability to support amoebic growth. After culturing on this bacterium, *Acanthamoeba* spp. exhibit stratified growth in which numerous cell layers exist and thus any inhibition by cell contact is apparent. In this way, the number of amoebic trophozoites in only one plate, with this bacterium, is about 50 times higher than in cultures with *A. aerogenes*. This luxuriant growth is very useful for initial isolations (because it represents fast growth) and for obtaining high cell densities for additional manipulations.

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Cryopreservation of *Trichinella* Newborn Larvae

P. Rossi and E. Pozio, Laboratorio di Parassitologia, Istituto Superiore di Sanità, Viale Regina Elena, 299 00161 Roma, Italy

ABSTRACT: A technique for the cryopreservation of *Trichinella spiralis* newborn larvae is described. A similar procedure for muscle-stage larvae was not successful.

Parasitologists in recent years have been interested in developing cryopreservation protocols to overcome some of the constraints in maintaining parasites under laboratory conditions.

At present, there are techniques for cryopreserving adult male worms of *Onchocerca* (Towson and Ham, 1986, Tropical Medicine and Parasitology **37:** 117–120), microfilariae of *Onchocerca* (Minjas and Townson, 1980, Annals of Tropical Medicine and Parasitology **74:** 571–573), and *Wuchereria* (Owen and Anantaraman, 1982, Transactions of the Royal Society of Tropical Medicine and Hygiene **76:** 232–233), microfilariae and third-stage larvae of *Dirofilaria* (Lok et

al., 1983, Journal of Helminthology **57**: 319–324), *Brugia* (Ham and James, 1983, Transactions of the Royal Society of Tropical Medicine and Hygiene **77**: 815–819), and *Dipetalonema* (Lowrie, 1983, American Journal of Tropical Medicine and Hygiene **32**: 767–771), larvae of gastrointestinal nematode parasites of domestic livestock (Parfitt, 1971, Research in Veterinary Science **12**: 488–489; Campbell et al., 1972, Veterinary Record **91**: 13; James, 1985, Parasitology Today **1**: 134–139), *Schistosoma mansoni* schistosomula (James, 1981, Experimental Parasitology **52**: 105–116), sporocysts (Cohen and Eveland, 1984, Journal of Parasitology **70**: 592), and eggs (Oliver-Gonzales and Vasquez, 1983, Journal of Parasitology **69**: 277–279).

The isoenzymatic identification of *Trichinella* strains carried out in our laboratory (Pozio, 1987, Tropical Medicine and Parasitology **38**: 111–116), required the development of a method for cryopreserving *Trichinella* isolates, in order to avoid *in vivo* passages that may cause changes in their biological characteristics (Dick, 1983, *In Trichinella* and trichinosis, W. C. Campbell (ed.), Plenum Press, New York and London, pp. 31–73).

We succeeded in cryopreserving *Trichinella* newborn larvae in our laboratory. First, the muscle-stage larvae (MSL) were assayed to determine: (1) the toxicity of common cryoprotectants at various concentrations and times of exposure (cryoprotectants included: dimethyl sulphoxide [DMSO], polyvinylpyrrolidone, ethanediol, and hydroxyethyl-starch); and (2) the viability and infectivity of cryopreserved MSL. We followed protocols described in the literature (James, 1985, loc. cit.).

DMSO at 10% concentration, in which larvae were incubated for 30 min at 37 C, proved to be the most suitable cryoprotectant. After thawing in a water bath at 37 C, MSL were viable, motile, and apparently not damaged, but they were not able to infect mice or rats. We also transplanted cryopreserved MSL directly into the rat duodenum in order to avoid the gastric barrier, but unsuccessfully. Finally, we tried to cryopreserve encapsulated MSL, but they were not viable after thawing.

Newborn larvae were obtained from adult worms (*T. spiralis* strain isolated in the U.S.A. from domestic pig, code: MSUS/US/50/ISS4) recovered from the small intestine of infected rats on day 6 after infection, by placing the gut in physiological saline solution for 90 min at 37 C. Adults were then washed 10 times in sterile physiological saline solution and cultured in 25-ml flasks in BME (Basal Medium Eagle) with Earle's salts, 10% foetal calf serum (FCS), 1% glutamine, 1% nonessential amino acids, 10,000 IU/ml penicillin, 7,500 IU/ml streptomycin, and 300 μg/ml 5-fluorocytosine at 37 C in 5% CO_2 atmosphere. After 18 hr, newborn larvae were separated from adults on 70-μm mesh filter and divided into 2 stocks containing the same number of larvae. The first of them was inoculated into control mice via the caudal vein, the second one was incubated in a freezing medium (culture medium with 10% FCS and 10% DMSO) for 15 min at 37 C in 1.5-ml plastic ampoules containing 10,000 newborn larvae in 1 ml of freezing medium per vial. The tubes were then placed in Goodwin and Thiel's slow cooling device (Goodwin and Thiel, 1967, Transactions of the Royal Society of Tropical Medicine and Hygiene **61**: 134–135) and cooled at 0.3 C min^{-1}. When −80 C was reached, the tubes were plunged into liquid nitrogen for storage. After a period ranging from 2 to 12 months the larvae were thawed by rapidly stirring ampoules in a water bath at 37 C, then placed in a 10× volume of the culture medium, in order to dilute the DMSO, and incubated at 37 C in CO_2 atmosphere for 3 hr. An average of 80% of newborn larvae were motile after thawing. After concentrating the larvae by centrifugation at 2,000 rpm for 10 min, they were injected into mice via the caudal vein. On day 25 after injection, the mice (including controls) were sacrificed and their muscles were ground in a grinder for 1 min in artificial gastric juice (1% w/v pepsin 10,000 IU, 1% v/v HCl in tap water) and then placed in a beaker containing artificial gastric juice for 2 hr at 37 C with magnetic stirring. After the artificial gastric juice was removed, the MSL were counted. In control mice, an average of 24% of newborn larvae developed in MSL. In mice inoculated with cryopreserved newborn larvae an average of 9% of newborn larvae developed in MSL, i.e., 38% in comparison with those obtained from control mice.

This method allows also the storage of a large number of parasite isolates and the establishment of a cryobank for *Trichinella* strains.

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Recovery of *Leishmania* (*Viannia*) *braziliensis* from Inoculated Hamsters

Norman E. Peterson, Maria A. F. Costa, Júlio A. Vexenat, Eduardo M. Netto, Philip D. Marsden, Albino V. Magalhães, and Air C. Barretto, Núcleo de Medicina Tropical e Nutrição, Universidade de Brasília, 70.910 Brasília, Brazil

ABSTRACT: *Leishmania* (*Viannia*) *braziliensis* has never been isolated from wild animals although it is apparently capable of inducing infections in man, dogs, and donkeys. An analysis of the standard hamster culture system for analyzing infectivity of *Leishmania* sp. was undertaken. Results indicate that for *L.* (*V.*) *braziliensis,* routine cultivation of aspirates taken from the inoculation sites of 1-mo-infected hamsters should be undertaken. Moreover, in at least 1 of the 3 strains examined, isolation of the parasite was only achieved after 84 days of cultivation.

Leishmania (*Viannia*) *braziliensis,* following Lainson and Shaw (1987, *In* The leishmaniases in biology and medicine, Vol. 1, W. Peters and R. Killick-Kendrick (eds.), Academic Press, London, pp. 1–120), has frequently been recovered from infected patients (Cuba et al., 1980, Boletin de la Oficina Sanitaria Panamericana **89:** 195–208; 1986, Transactions of the Royal Society of Tropical Medicine and Hygiene **80:** 456–457), dogs (Barretto et al., 1982, Resumos do IX Reunião Anual de Pesquisa Basica em Doença de Chagas, Caxambu, Minas Gerais, p. 109), and a donkey (Vexenat et al., 1986, Memórias do Instituto Oswaldo Cruz **81:** 237–238) from southeastern Bahia, Brazil, by hamster inoculation or culture. However, it has yet to be isolated from a wild animal even though more than 1,000 have been examined by inoculation in hamsters or Difco blood agar media (Walton et al., 1977, Journal of Parasitology **63:** 1118–1119), or both. The main method of screening these wild animals has been by inoculating tissue from the liver and spleen and skin from the nose and tail of the wild animal into the hind feet and peritoneal cavity of a hamster. The hamsters were then examined at weekly intervals for an ulcer at the inoculation site, loss of pelage, or other abnormality. If erythema, swelling, or other abnormality was observed, aspirate from the lesion was inoculated into culture media. Giemsa-stained smears of the aspirate were examined for amastigotes also. On sacrificing the hamster 18–24 mo after inoculation, the liver, spleen, and inoculation site were examined by cultivating tissue homogenates in Difco blood agar and examining stained smears. We have recovered a parasite very similar to *Leishmania* (*Leishmania*) *amazonensis* from the spiny rat *Proechimys iheringi* (Barretto et al., 1985, Revista da Sociadade Brasileira de Medicina Tropical **18:** 243–246), demonstrating that the procedure works with this subgenus of *Leishmania.*

Our failure to isolate *L.* (*V.*) *braziliensis* led us to study the development of this species in hamsters inoculated with amastigote-rich tissues using Giemsa-stained smears and culture as detection methods.

Two experiments were conducted. In the first, the behavior of MHOM/BR/83/LTB-300, a strain of *L.* (*V.*) *braziliensis* well characterized by isoenzyme, DNA, and monoclonal analysis, was examined. Pairs of hamsters were sacrificed weekly over 7 wk after inoculation either into both hind feet (group A animals) or intraperitoneally (group B animals) with an amastigote-rich suspension from hamster tissues. In group A animals, aspirates from the inoculation site, inguinal lymph gland, tibia ossea of the left hind leg, liver, and spleen were examined. In group B animals, only the liver and spleen were examined. All aspirates were examined in 2 ways. First, an aspirate was inoculated into paired tubes of culture media maintained at 26 C and examined weekly for 4 wk. After this period, the cultures were considered negative if they contained no parasites. Second, Giemsa-stained smears were prepared from the aspirate and screened for amastigotes. The right hind foot of group A animals was fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin for histopathological study. Liver and spleen sections were similarly examined.

In the second experiment, biopsies from the skin ulcers of 3 patients, 2 from Três Braços, Bahia, located 75 km southwest of Valença (MHOM/BR/86/LTB-865 [group I] and

MHOM/BR/LTB-866 [group II]), and 1 from Corte de Pedra located 45 km southwest of Valença (MHOM/BR/86/LTCP-652 [group III]), were triturated in phosphate-buffered saline (Na_2HPO_4 34.17 g, NaH_2PO_2 0.68 g, NaCl 4.25 g, and H_2O 1,000 ml) containing 16% glycerol and inoculated into both hind feet and the peritoneum of 5 hamsters for each patient. *Leishmania* (*V.*) *braziliensis* was recovered from all 3 patients and characterized by immunofluorescence using monoclonal antibodies.

Aspirates from the inoculation site (hind foot) were taken weekly and seeded into Difco blood agar culture media. One animal from group I and 1 animal from group II were sacrificed on day 24 postinoculation and tissues from the livers and spleens were seeded into separate tubes of culture media. Hepatic punctures from the surviving hamsters were taken periodically between days 24 and 95 postinoculation and seeded into culture media. The inoculated culture tubes were maintained and examined as described in the first experiment.

In the first experiment, using the laboratory strain of *L.* (*V.*) *braziliensis* (LTB-300), parasites were recovered by the end of the first week from the hind-foot inoculation site (group A), and in subsequent animals this site was always positive for the duration of the experiment (7 wk). Visible swelling of the inoculation site was present at 1 wk. Although histology showed the chronic inflammatory infiltrate of cutaneous leishmaniasis at this site in all 7 animals, parasites were only visible on necropsy up to week 5.

In both animal groups (group A and group B), parasites were detected by culture in the liver and spleen 2 wk after inoculation. In the group A animals, parasites were also present in the lymph gland draining the inoculation site and the medulla ossea of the tibia. Subsequent rates of positive culture from these sites in weeks 3–7, inclusive, were as follows: group A animals, inguinal lymph gland 2/4, medulla ossea 3/4, liver and spleen 3/4; group B animals, liver and spleen 3/4.

No amastigotes were seen in liver sections, although on 2 occasions (weeks 2 and 7) they were detected histologically in the spleen of group A animals.

These results were unexpected in terms of the rapid development of signs observed at the inoculation site and parasite detection there and in the viscera. In view of the fact that LTB-300 had been passed through hamsters several times prior to the experiment, it was thought that these results might reflect selection or adaptation of the strain to rapid growth in hamster tissues. Therefore, the behavior in hamsters of isolates taken from patients with acute cutaneous leishmaniasis living in Três Braços (LTB-865 and LTB-866) and Corte de Pedra (LTCP-562) were examined. The inoculation site aspirations from these hamsters were cultured on the 17th day after inoculation, and all 10 hamsters inoculated with LTB-865 (group I) and LTB-866 (group II) were positive. There was visible swelling at the inoculation site. One animal from each of the 2 groups was sacrificed 24 days after inoculation, and both liver and spleen cultures were positive. Periodic hepatic punctures, using an intramuscular needle, demonstrated parasites from 24 to 51 days in all but 1 of the 8 remaining hamsters. This possibly reflects difficulty in obtaining sufficient material, as liver tissue from 1 hamster inoculated with LTB-866 had a positive culture at necropsy at 102 days, after 5 previous liver aspirations taken on days 24, 38, 51, 79, and 95 were persistently negative.

The behavior of isolate III (LTCP-562) was different. No physical signs were observed at the inoculation site and no parasites were isolated from it on day 17, but 3 of the 4 surviving animals (1 hamster died within a week of being inoculated) produced positive aspirates from this site on day 84. Liver aspirates from these 3 hamsters were negative at 14, 28, 41, 69, and 85 days postinoculation, and when necropsied at day 92, they had no detectable parasites in their livers and spleens. The fourth hamster produced positive liver aspirates at days 28 and 64. It died on day 84.

The following observations can be made regarding these preliminary experiments:

1) A laboratory-maintained isolate, LTB-300 and 2 of 3 field isolates of *L.* (*V.*) *braziliensis* produced visible edema at the inoculation site within the first month and parasites could be isolated into culture.
2) In 1 field isolate, LTB-562, parasites were only detected in the inoculation site after 84 days, and no visible signs were observed there. Parasites were detected in the liver of only 1 of the 4 hamsters.
3) Liver aspirates taken and cultured after 1 and 2 mo often produced living parasites.
4) All cultures of hind-foot (inoculation site) aspirates produced parasites.

5) The results of the second study indicate that cultures of aspirates from the hind foot (inoculation site) of hamsters are more reliable for recovering *L.* (*V.*) *braziliensis* parasites than cultures of liver punctures or liver and spleen aspirates during necropsy.

Using laboratory-maintained human strains of *Leishmania braziliensis panamensis* inoculated into rodents (*Tylomys panamensis*), Thatcher et al. (1965, Journal of Parasitology **51**: 842–844) recovered parasites from inoculation sites after about 3 wk. Grimaldi et al. (1984, Transactions of the Royal Society of Tropical Medicine and Hygiene **78**: 560) were able to culture 36 of 40 primary isolations of *L.* (*V.*) *braziliensis* from the inoculation site of hamsters within 3 wk postinoculation of biopsies from human lesions. In view of these observations and our results reported above, the routine cultivation of aspirates from the inoculation site of hamsters 1 mo after being inoculated with tissue suspected of containing *L.* (*V.*) *braziliensis* is indicated. However, it should be noted that in 1 of the 3 strains examined, isolation was only achieved after 84 days. This suggests that strains of *L.* (*V.*) *braziliensis* exist in our endemic area that grow more slowly than others in hamster tissue.

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Refractoriness of *Callithrix penicillata* Red Blood Cells to *Plasmodium falciparum In Vivo* and *In Vitro*

Eliana M. M. Rocha*†, Leogenes H. Pereira*, Virgílio E. do Rosário‡, and Antoniana U. Krettli*†§, *Department of Parasitology, University of Minas Gerais, †Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, and ‡Instituto Evandro Chagas, Belém. §To whom correspondence should be addressed

ABSTRACT: Attempts to infect the New World marmot *Callithrix penicillata* with *Plasmodium falciparum* were unsuccessful. Attempts were also made to infect red blood cells of *C. penicillata* and *Saimiri sciureus* with *P. falciparum in vitro*, and these too were unsuccessful due to a high rate of hemolysis produced by apparently adverse culture conditions. It is concluded that modifications to the existing culture conditions will need to be made before successful parasitemia can be induced *in vitro* in simian erythrocytes.

Plasmodium falciparum has been successfully adapted to some simian species of the New World like *Aotus trivirgatus* (Geiman and Meagher, 1967, Nature **215**: 437–439) and *Saimiri sciureus* (Young and Rossan, 1969, Transactions of the Royal Society of Tropical Medicine and Hygiene **63**: 686–687). Since both are only available in the Amazon Region and are difficult to rear in captivity, we attempted to infect the New World marmoset *Callithrix penicillata*, which is widely distributed in our region (Minas Gerais State, Brazil), less expensive to maintain in comparison with other primates, and of small size ([illegible]0–400 g) and therefore easy to handle. Furthermore, it is being reared indoors at our animal facilities. This animal is susceptible to *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Leishmania*, and *Toxoplasma gondii* infections (Silva, 1974, Susceptibility of marmosets of the genus *Callithrix* to some parasite protozoa of man [in Portuguese]. Masters Thesis. University of Minas Gerais, Belo Horizonte, MG, Brazil, 73 p). However, the author did not succeed in infecting 2 intact *C. penicillata* using blood from a patient with *P. falciparum* malaria. In a new attempt to establish infection in this species, we have used a strain of *P. falciparum* previously adapted to the simian *A. trivirgatus* and maintained in *S. sciureus* (Palo Alto strain) and various strains maintained *in vitro* in continuous cultures. The *C. penicillata* were submitted to high concentration inocula, immunosuppression by cyclophosphamide (10–75 mg/kg i.m.), and supplementation with p-aminobenzoic acid (PABA). Thirty-one inoculations were performed in the following marmosets: 5 intact, 19 splenectomized, 3 drug-immunosuppressed before and after inoculation, and 4 splenectomized and treated with cyclophos-

phamide. A special diet supplemented with PABA was offered to 6 splenectomized and to 1 intact *C. penicillata.* Four *S. sciureus* were used as donors of the Palo Alto *P. falciparum* strain. The marmosets were inoculated with 10^5–10^9 infected red blood cells (RBC) by intraperitoneal, intracardiac, or intravenous injection. Blood smears, made immediately after inoculation and daily thereafter, up to day 90, were Giemsa stained and examined to follow the course of parasitemia. *Callithrix penicillata* did not develop parasitemias regardless of the parasite origin, size of inocula, or route of inoculation. Four animals receiving very high inocula (10^9/i.v.) of the Palo Alto strain showed circulating parasites in blood smears for up to 5 days after inoculation and then became negative. Moreover, the refractoriness of *C. penicillata* to *P. falciparum* was not influenced by addition of PABA to the diet, splenectomy, or cyclophosphamide. In contrast, 4 specimens of *S. sciureus* developed high parasitemias and lethality when inoculated with the Palo Alto strain. These data support previous work on the susceptibility of *S. sciureus* to *P. falciparum* (Gysin et al., 1980, Journal of Parasitology **66:** 1003–1009) and confirm the virulence of the Palo Alto strain we used.

Attempts were made to infect RBC of *C. penicillata* and *S. sciureus in vitro.* The simian RBC were introduced into *P. falciparum* cultures with the human RBC either gradually or at once and maintained as described by Trager and Jensen (1976, Science **193:** 673–675). To avoid agglutination of the simian RBC, the human sera were preadsorbed with the respective primate RBC overnight. As expected, the control cultures with human RBC had increasing parasitemias. However, the *P. falciparum* did not grow in the simian erythrocytes, partly because of hemolysis, which occurred seemingly as a result of the great osmotic fragility of these erythrocytes under culture conditions. Recent results obtained by Fandeur and Dedet (1986, Memórias do Instituto Oswaldo Cruz **81:** 165–170) showed that *P. falciparum* could be maintained in *S. sciureus* RBC for no longer than 19 days *in vitro* with low parasitemias, and apparent erythrocyte lysis as well. Therefore, it seems likely that modifications of *in vitro* culture conditions to avoid hemolysis of simian erythrocytes will be necessary before such attempts can be successful.

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BOOK REVIEW . . .

Symbiosis: An Introduction to Biological Associations, by Vernon Ahmadjian and Surindar Paracer. University Press of New England (Publishers), Hanover and London. 1986. xii + 212 p.

This small book of 212 pages is an attempt to cover a very large subject. *Symbiosis* as used here means the living together of two or more different species of organisms in a relatively intimate association. Hence it includes parasitism as well as mutualism and commensalism. The book is organized essentially on a taxonomic basis, as is evident from the chapter headings. Only chapters 1 (Introduction), 5 (on Symbiosis and the origin of the eukaryotic cell), and 13 (on Symbiosis and evolution) are of a general comparative nature. The other chapters treat: Viral symbiotic associations; Bacterial associations with other bacteria, protoctists, and animals; Bacterial associations with plants; Fungi as symbionts of protoctists and animals; Fungi as symbionts of fungi, algae, and plants; Parasitic and mutualistic protozoa; Algal associations; Helminthic associations; Plant symbiotic associations; and Behavioral and social symbiosis. Each chapter cites briefly several particularly interesting or important examples of the kind of association being considered. In the section on fungal–algal associations it is good to have fairly detailed discussion of lichens—fascinating organisms with which Dr. Ahmadjian has himself done much original work, and which are all too often forgotten about. At the end of each chapter there is a set of "Review questions" followed by a short useful list for "Further reading" and a brief "Bibliography." The book is nicely printed with attractive line drawings and a small number of photomicrographs and electron micrographs. It is unfortunate that none of these micrographs has any indication of magnification.

I agree whole-heartedly with the authors that symbiosis is a phenomenon of major biological significance and should occupy a correspondingly major place in the biological curriculum. A book like this, attempting to bring together all the varied aspects of this large field, could indeed be useful. My first impression on looking through the book was a favorable one. On reading it in detail, however, I was disappointed. There is too little on the functional interrelationship between the associated organisms and too much emphasis on definitions and special terms. What really is gained with a term like "mycophycobiont" rather than fungal–algal association? Yet despite this emphasis on a superficial kind of precision, there are numerous errors of fact and interpretation. I will cite only a few. On p. 8, in discussing the location of symbionts, the authors state that most "endosymbionts," by which they mean intracellular symbionts, are separated from the cytoplasm by the "host plasma membrane" and therefore are "outside the host cell." It is true that many kinds of intracellular organisms are surrounded by a membrane derived originally from the host cell. But in the few instances where this membrane has been adequately studied, it has been found to be very different from the host plasma membrane. The symbiont is no more "outside" the cell than is the cell's nucleus. Again on p. 8, it is stated that "In cases of parasitism the symbiosis persists throughout the host's life." Some parasites are indeed very persistent, but full recovery, even from malaria for example, does often occur without treatment. On p. 54, it is wrong to state that mycoplasmas cannot be grown in pure culture. A number of these have been so grown. On p. 109, one finds this misleading statement: "Asexual reproduction is by binary or multiple fission and is called *schizogony*." Schizogony and binary fission are quite different. This error probably results from a futile attempt to describe a "generalized life cycle" for parasitic protozoa with a useless diagram. Yet the first species considered is *Entamoeba*, which does not at all fit that life cycle. On p. 115 it is said that intestinal flagellates "do not reproduce sexually." Have the authors forgotten the beautiful work of L. R. Cleveland on the sexual cycles of the symbiotic flagellates of *Cryptocercus*? There is no mention anywhere in the book of this outstanding study and of its relevance to hormonal cycles of the host. But on p. 119 and again on p. 129, there is perpetuation of the myth that cellulose digestion by termite flagellates "may be the result of intracellular bacterial symbionts." There is no longer any need for such a statement, since M. Yamin has grown at least two species of termite flagellates in axenic culture and shown their ability to utilize cellulose. It is with regret that I conclude that I cannot give this book the high recommendation that its subject deserves.

Dr. William Trager, Rockefeller University, 1230 York Ave., New York, New York 10021-6399.

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ANNOUNCEMENT . . .

Disease and Management of Threatened Bird Populations: Register of Laboratories and Reference Centres

The International Council for Bird Preservation is publishing the Proceedings, edited by J. E. Cooper, of the Symposium on Disease and Management of Threatened Wild Bird Populations held in Canada in 1986. With growing interest in, and appreciation of, the importance of disease and wildlife conservation, it was agreed to include a register of persons and organisations working on all aspects of avian pathology and disease. We are most anxious to include all those interested in being consulted over problems relating to morbidity and mortality and to facilitate exchange of information and material between those working on avian disease.

Anyone who would like to be listed in the register please write as soon as possible for details and a questionnaire to: N. Hillgarth, Department of Zoology, South Parks Road, Oxford, England OX1 3PS, or J. E. Cooper, Royal College of Surgeons of England, 35/43 Lincoln's Inn Fields, London, England WC2A 3PN.

J. Parasit., 74(3), 1988, p. 517

ANNOUNCEMENT . . .

Call for papers

A 10-speaker symposium entitled "Avian responses to parasitism" will be held during the meeting of the American Ornithologists' Union 15–18 August 1988 in Fayetteville, Arkansas, USA. Though speakers for the symposium have been chosen, supplemental papers on bird–parasite interactions are sought for the general sessions. Additional papers will be considered for a proposed symposium volume. For further information contact Dale H. Clayton, Committee on Evolutionary Biology, University of Chicago, 1025 E. 57th St., Chicago, Illinois 60637 (312-684-6277) or Jenella Loye, Department of Biology, University of Utah, 201 Biology Building, Salt Lake City, Utah 84112 (801-581-6517).

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1988 MEETING OF THE AMERICAN SOCIETY OF ZOOLOGISTS

and

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Chemical Factors that Influence the Settlement and Metamorphosis of Marine Invertebrate Larvae
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